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PLANT PHYSIOLOGY

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VOLUME 33

1958

THE SCIENCE PRESS

LANCASTER, PENNSYLVANIA

114336

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PLANT PHYSIOLOGY

VOLUME 33 JANUARY, 1958 NUMBER 1

MOVEMENT OF CHLORINE WITHIN PLANTS[1]

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For some years a project directed toward study of the movement of materials into and through higher plants has been actively studied in the Department of Soils and Plant Nutrition (3, 4, 6). Much has been learned of the absorption of halide by abscised roots and intact plants from inorganic solution cultures. Recent observations on the essentiality of chlorine in nutrition have posed the problem of movement of this element within higher plants (11). That halide is rapidly absorbed from solutions by roots and moves to shoots is well known from exudation and other studies (3, 4, 6). While this primary movement (mobility and translocation are implied) is certain, less is known of the secondary movements or the remobility and retranslocation of halide within the plant body. It is the object of this report to present data bearing on the remobility of chlorine as evidenced by retranslocation of this element, such retranslocation being observed through a chlorine distribution pattern which changes with time.

Though some observations here bear on the initial absorption of chlorine into the plant and translocation to various plant parts, others bear on the removement within the plant body. Those observations dealing with measurement of the retranslocation of chlorine are regarded as being especially pertinent. Retranslocation is here defined as follows: *Retranslocation of a substance in a higher plant is said to occur when the substance moves out of a terminal location or organ into which it previously had moved from some other part of the plant.*

Retranslocation leads, with time, to an observable redistribution, the pattern of which is necessarily dependent upon all factors which influence movements of substances within plants. By this definition of retranslocation, chlorine, reaching a leaf by movement from the root, would not be said to have been retranslocated. Nor would chlorine, reaching a root from a leaf to which it had been externally applied, be said to have been retranslocated. But if some of this same (leaf-applied) chlorine were demonstrated to have subsequently moved from the root to the shoot growing point, such chlorine would be said to have been retranslocated.

It is possible to estimate the movement of an element by several different methods. For instance, it would seem logical to evaluate retranslocation in terms of the localization of symptoms of a deficiency of the element in question (1, 14). By use of this technique, one would probably decide that boron, calcium, and iron were not readily retranslocated, since deficiency symptoms of these elements occur first in young tissues of the plant. Magnesium and molybdenum, by this classification, would appear to be very readily retranslocated. Application of this method to the consideration of chlorine movements should give intermediate results, since chlorine deficiency symptoms usually appear first on recently matured leaves, rather than on either the oldest or the youngest leaves (14). Conclusions such as these might be useful as approximations, but would ignore possible differences between types of tissues in requirement for the element in question. Further, as was shown by Oserkowsky (9), some elements, such as iron, might actually be present in abundance in a tissue showing deficiency symptoms.

Another method of estimating movement is by means of radioautography of plants to which radioactive isotopes of the element have been applied. By this technique, Biddulph (1, 2) obtained the mobility sequence, $P > S > Fe > Ca$. It is doubtful that the symptomatic and radioautographic methods of approach would measure the same phenomena, even though the results might agree in many instances. The symptomatic approach would be, at its best, a crude measure of the ability of younger (newer) tissues to obtain a required element from older tissues which had acquired the element by primary translocations. The radioautographic technique may measure in part the primary distribution of an element from a plant organ (root or leaf) to which the element has been externally applied. It is evident that Biddulph's radioautographs show a significant amount of movement even for his "least mobile" element, calcium.

Woodbridge (see Biddulph (1) experiment fig 7) observed changes in concentration of radiophosphorus in parts of bean plants over a 4-day period following a 4-day root application of P^{32}. During this period of observation the concentrations of P^{32} decreased in the roots, stem and lower leaves. It was concluded that considerable radiophosphorus was withdrawn from older tissue and moved to newly formed leaves indicating "a rather free mobility of P^{32} in the 'circu-

[1] Received May 14, 1957.

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latory systems' of the plant." Since nonradioactive phosphorus as phosphate was presumably supplied during the secondary interval, some radiophosphorus may have been displaced, so as to enhance the observed mobility. Further, it seems that possible losses from roots and stem to distal leaves may have included, at least in part, P^{32} which was in the course of longitudinal movement. Concentration decreases in opposite leaves, proximal alternate trifoliate leaves, stem and roots might be attributable, at least in part, to dilution through growth enlargement. Certainly the newer formed distal leaves contained high concentrations of P^{32}, but total values of activity in the various plant fractions would have yielded a more conclusive evaluation of redistribution.

Watson and Petrie (12) and Williams (13) also analyzed plant parts at different times during growth, observing the progressive redistribution patterns of nitrogen and phosphorus. Their results are graphically expressed as changes of the total content of the element in the plant. Williams found a decrease of nitrogen or phosphorus in one or more vegetative organs of oats accompanied by an increase of the element in the inflorescence. Retranslocation was indicated. Watson and Petrie observed retranslocation of nitrogen in tobacco plants from older leaf groups to those younger in the acropetal series, and from leaves to reproductive organs, during development.

In the present work, the radioautographic method of approach to retranslocation has been applied to chlorine. Further, the progressive redistribution of chlorine has been followed in ontogenetic fractions of the various plant organs, where feasible. The data are expressed graphically in net total terms rather than as concentration changes in the plant fractions, although some of the data are also expressed as concentrations. Also, the supply of test element has been limited in order to more accurately define that part subject to retranslocation.

Recent experiments of Johnson et al (8) and of Ulrich and Ohki (11) have indicated that plants which are low in chlorine usually obtain relatively large proportions of their chlorine content from unknown sources, possibly the atmosphere. In view of this fact, it was considered that any studies of movement of chlorine in plants, especially at levels approaching chlorine deficiency, would have to be carried out with radioactive chlorine. Radiotracer methods would serve to distinguish the applied chloride from the (possibly much greater) inadvertent contamination.

Materials and Methods

Two experiments were undertaken to ascertain the extent to which retranslocation of chlorine occurs in higher plants. In the 1st, radiochlorine was applied to the leaves of plants and was later detected by means of radioautography of whole plants and by use of a Geiger counter on plant parts. In the 2nd experiment, radiochloride was supplied in the culture solution to the roots of plants and was later measured in various plant parts by means of a Geiger counter. This technique was similar to that used by Williams (13) and by Watson and Petrie (12), but with modification to preclude the possibility that leaf chlorine increases might be at the expense of the nutrient solution, rather than of the older leaves.

Experiment 1: Sugar beet and tomato seeds were germinated on moist cheesecloth at the surface of a 1/80 strength macro-nutrient solution (8) and, after 9 days, were transferred to two-liter Pyrex beakers which had paraffined plaster-of-Paris lids. The roots were shielded from light and the solutions were aerated continuously.

The experiment was carried out in a glasshouse which had charcoal and limestone filters on the air intakes. These filters prevented smog damage to plants and possibly reduced the amount of airborne chlorine in the glasshouse atmosphere.

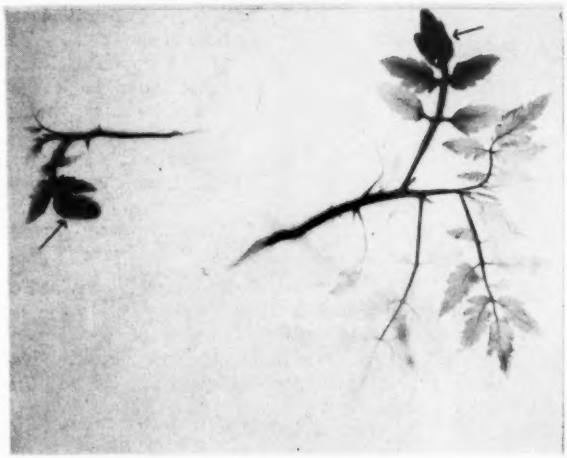
The full-strength nutrient solution contained the following salts in millimoles per liter: KNO_3, 6; $Ca(NO_3)_2$, 4; $NH_4H_2PO_4$, 2; $MgSO_4$, 1; KCl, 0.050; H_3BO_3, 0.025; $MnSO_4$, 0.005; $ZnSO_4$, 0.002; $CuSO_4$, 0.0005; and H_2MoO_3, 0.0001. Iron was supplied by a piece of steel wire extending from top to bottom inside of each beaker.

Five plants were transplanted to each of several two-liter beakers and were subsequently thinned to two similar plants per beaker. Two weeks after transplanting, one beaker of tomato plants was selected for uniformity of the two plants. At 6 P.M. the terminal leaflet of the 3rd true leaf of each of the plants was submerged in 100 ml of a solution containing 809 μg atoms of labeled chlorine as NaCl in a 0.05 % solution of a neutral detergent (Vel). At 8 A.M. the next day the leaflets were removed from the radiochloride solution and were rinsed five times by immersion in distilled water. The plants were left for 24 additional hours under their normal culture conditions, after which one of the plants was removed from the culture solution. The roots of this plant were rinsed with distilled water and blotted with cleansing tissue. The plant was placed in a press and air-dried. One week after the 1st plant had been removed, the 2nd plant was similarly harvested, pressed and dried. The two plants were then placed together on an x-ray film so as to provide a radioautograph (fig 1).

The same technique was used with one pair of sugar beet plants, about 5 sq cm of the tips of the 4th leaves being dipped into the radioactive solution. The resulting radioautograph is shown as figure 2.

Experiment 2: Marglobe tomato seeds were germinated as in experiment 1. The nutrient solution was the same as that used in experiment 1 except as is stated below.

All nutrient salts except the molybdic acid were purified by two recrystallizations from Pyrex-distilled water. The molybdic acid was prepared from ammonium molybdate which conformed to ACS specifications, and was not further purified. Analyses of similarly prepared salts have shown that the chloride



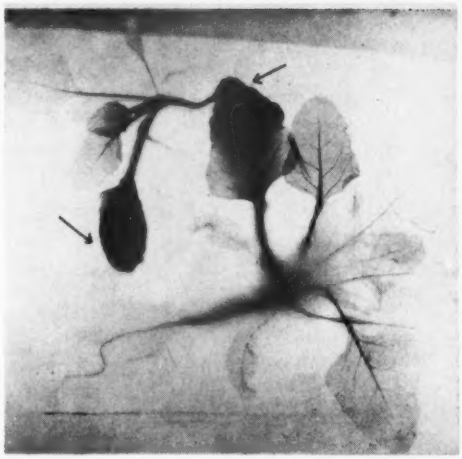


FIG. 1 (*left*). Radioautographs of leaf-applied Cl^{36} in tomato plants, one day (smaller plant) and 8 days (larger plant) after application. Treated leaflets are marked with arrows.

FIG. 2 (*right*). Radioautographs of leaf-applied Cl^{36} in sugar beet plants, one day (smaller plant) and 8 days (larger plant) after application. Treated leaflets are marked with arrows.

supplied by the total amounts of the salts used would be less than three μg atoms per culture.

The seedlings were transplanted into half-strength culture solution in four-liter beakers on June 8, 1956. Subsequent additions of nutrients, as well as harvest dates, are calculated from this date. A 2nd half-strength portion of nutrients was added on the 19th day, and full-strength portions were added on the 36th and 44th days. Chloride was supplied only once, on the 5th day. Twelve of the beakers were designated as deficient-chloride cultures, each receiving 38.5 μg atoms of chlorine as HCl labeled with 0.5 μc of Cl^{36}. The remaining 12, the adequate-chloride cultures, each received 77 μg atoms of chlorine as HCl labeled with 1.0 μc of Cl^{36}, and 200 μg atoms of stable chloride as KCl. Labeled chloride was available continuously in the culture solution until exhausted by absorption, a period of approximately 31 days; the 40- and 55-day harvests, then, were of plants which had been growing (since the 31st day) in a medium essentially free of chlorine.

At the time of harvest the plants were cut into the designated fractions, placed in paper bags, and dried at 85° C. The fractions were weighed and ground, and aliquots of this ground material were weighed into planchets. The radioactivity was measured with a 2 mg/cm^2 end-window Geiger tube, and the amounts of radiochlorine were calculated by comparison with standards made from similar non-radioactive (except for natural radioisotopes) plant material and known amounts of Cl^{36}.

The plants were thinned to two per beaker on the 11th day. The plants removed by this thinning were grouped together into four lots, two from the adequate-chloride cultures and two from the deficient-chloride cultures. Radioactivity measurements were made on these and the average value per beaker is shown as "thinnings" in figures 3, 4, 5 and 6. At each harvest date two adequate-chloride cultures and two deficient-chloride cultures were harvested. Only cultures which appeared to be representative of the entire set were taken for harvest.

The 1st harvest was made 19 days after the transplanting date, when the 6th leaf was the smallest readily discernible leaf on each plant. The plants of one adequate-chloride and one deficient-chloride culture were harvested intact (figs 5 and 6, respectively) while the plants of another culture at each chloride level (figs 3 and 4, respectively) were separated into fractions, as follows:

Roots	All portions of the plants below the cotyledons.
Stems (0–6)	The main axis of the plant from the point of attachment of the cotyledons upward, petioles and petiolules of leaves growing directly from the main axis, and the terminal bud.
Laminae (0–6)	Laminae of all discernible leaves growing from the main axis. Also cotyledons.

The 2nd harvest was made 12 days later (31 days after transplanting) when the smallest readily discernible leaf was the 11th. At this time all of the harvested plants were divided into four fractions, as follows:

Roots	All portions of the plants below the cotyledons.
0–6	Main axis and associated leaves from the cotyledons to just above the 6th leaf. This fraction contained only struc-

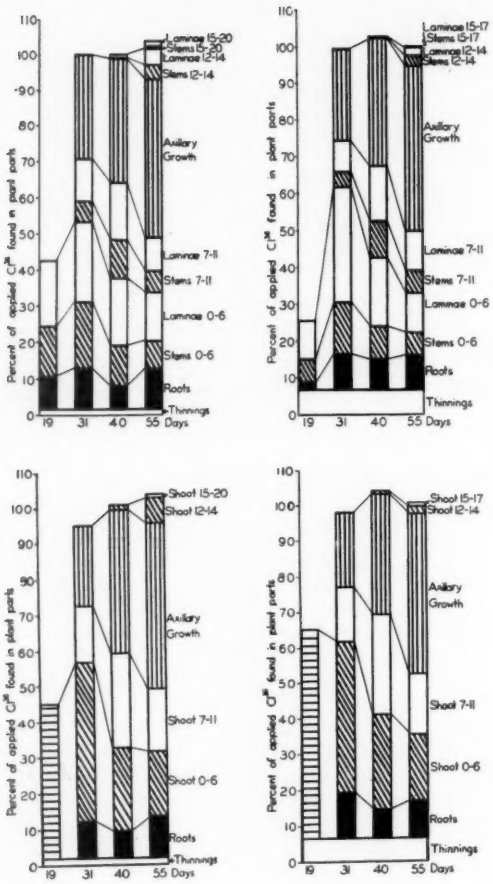


FIG. 3 (*top left*). Labeled chlorine contents of subdivided tomato plant fractions expressed as percentages of applied labeled chloride. Chlorine supply (277 μg atoms) was adequate for maximal plant growth.

FIG. 4 (*top right*). Labeled chlorine contents of subdivided plant fractions expressed as percentages of applied labeled chloride. Plants received only 38.5 μg atoms of labeled chlorine, and showed chlorine deficiency symptoms after the 31st day.

FIG. 5 (*bottom left*). Labeled chlorine contents of unsubdivided plant fractions expressed as percentages of applied labeled chloride. Unlabeled bar, horizontally marked for 19-day harvest, represents whole plant. Chlorine supply (277 μg atoms) was adequate at all times for maximal plant growth.

FIG. 6 (*bottom right*). Labeled chlorine contents of unsubdivided plant fractions expressed as percentages of applied labeled chloride. Unlabeled bar, horizontally marked for 19-day harvest, represents whole plant. Plants received only 38.5 μg atoms of labeled chlorine, and showed chlorine deficiency symptoms after the 31st day.

tures that were present at the time of the first harvest.

7–11 — Main axis and associated leaves from just above the 6th leaf up to and including the tip of the plant.

Axillary growth — Lateral stems and the leaves growing from them, flower buds, flowers, and fruits (not all structures were present at the second harvest).

The 7-11 fraction and axillary growth consisted of material which had developed subsequent to the 1st harvest. The 0–6 and 7–11 fractions of the plants of one adequate-chloride beaker and one deficient-chloride beaker were further subdivided into "stems" (including petioles and the terminal bud) and "laminae."

The 3rd harvest (made after 40 days, when the smallest readily discernible leaf was the 13th or 14th) was similar to the 2nd, but with the addition of 12–14 fractions representing new material since the 2nd harvest.

Similarly, the 4th harvest (made 55 days after transplanting, when the smallest discernible leaf on the main axis was the 16th to 20th) included 15–17 (deficient Cl) or 15–20 (adequate Cl) fractions, representing new material since the 3rd harvest.

RESULTS AND DISCUSSION

EXPERIMENT 1: From the distribution of radiochlorine in the smaller plant of figure 1, it can be seen that in the first 38 hours following the beginning of the 14-hour period of leaf dipping, chlorine reached young leaves, older petioles, the stem, and at least portions of the roots of the tomato plant. It is conceivable that this chlorine movement was the result of mass solution movement through and along cell walls and through the xylem, in accord with water gradients set up at some time during the 38-hour period. It seems likely that such cell wall and xylem chlorine would be exhausted by the end of this time so that movement occurring subsequent to the 38-hour harvest would probably be by usual modes of translocation. That such translocation took place can be seen from the radioautograph of the larger plant in figure 1. The treated leaflet had less radiochlorine than had the corresponding leaflet of the smaller plant, while the other parts of the larger plant had much more radiochlorine than did such parts of the smaller plant.

Figure 2 shows similar movements of leaf-applied radiochloride in sugar beet plants. Measurements of the radiochlorine in the plant fractions by means of a Geiger counter substantiated visual comparisons within figure 2. Only 19 % of the radiochlorine of the smaller plant was in parts other than the treated leaf and its petiole, while 40 % of the radiochlorine had moved from the treated leaf and corresponding petiole of the larger plant.

It is possible that some of the chlorine movement demonstrated in figures 1 and 2 was an artifact, as

described by Pallas and Crafts (10). Unnatural movement during drying was not investigated, but it seems unlikely that extensive movement of chlorine to all parts of the plant could have resulted from the drying process.

EXPERIMENT 2: The distributions of labeled chlorine in the different plant fractions at each harvest date are shown in figures 3 through 6. These amounts of labeled chlorine are expressed as percentages of the total applied labeled chloride. Excess over 100 % for any given harvest represents experimental error. Each vertical bar represents the sum of measurements on each of two tomato plants from one culture. The distributions for the plants which were not subdivided into "stems (and petioles)" and "laminae" (figs 5 and 6) show excellent agreement with those whose fractions were subdivided (figs 3 and 4, respectively).

It is evident from figures 3 through 6 that only part of the labeled chlorine had been absorbed by the plants during the first 19 days. Moreover, at this time there was great variation between replicates in the percentage absorbed. After 31 days, however, substantially all of the labeled chlorine had been absorbed, less than 0.1 % of the applied chloride remaining in the nutrient solution. Hence, labeled chlorine subsequently appearing in newer growth would necessarily have come from within the plant. Such a redistribution is shown for the 3rd harvest, at 40 days. The amounts of labeled chlorine in the newer portions of the plants increased considerably, while the labeled chlorine percentage in the older portions of the plants, notably the oldest 6 leaves and the accompanying stems and petioles, concurrently decreased. Similar results are evident for the redistribution between the 40- and 55-day harvests.

Much of this redistribution of labeled chlorine within the plant falls within the definition of "retranslocation" as used in this paper. It is true that chlorine, entering the new laminae 12–14 and laminae 15–20 fractions at the expense of the "stems" portion of the "stems (and petioles) 0–6" could not be said to have been necessarily retranslocated. Such chlorine might have been moving en route through the stems at the time of the 31-day harvest, and its subsequent appearance in newer tissues could be taken as a matter of course. Chlorine which moved into the newer tissues at the expense of the laminae 0–6, however, must have undergone retranslocation. Such chlorine had first moved into the roots, and from there had been translocated to the laminae of the first 6 leaves. Then the direction of movement was reversed, the chlorine moving out of the older leaves, into the stem, and finally into the newly formed tissues. Such chlorine did not become firmly bound in the laminae 0–6 (as perhaps might have been the case had calcium been used instead of chlorine), but was again mobile or free to move out of the laminae when conditions became proper for such movement.

Quantitative calculations from figure 4 (deficient-chlorine plants) indicate that the above was the case. By the 31st day, essentially all applied chloride had been absorbed. The deficient plants increased in dry weight from 15 to 89 g per culture between the 2nd and 4th harvest. Comparison of percentage distribution between plants at 31 and 55 days shows that the newer fractions (those depicted above laminae 7–11 in fig 4) had gained 29.7 % of the total labeled chlorine during this 24-day period. An essentially equal amount (28.4 %) of the total was lost from the older fractions. Of the 28.4 % moving from the older growth, 7.8 % was lost from non-terminal locations, e.g., the stems 0–6 fraction. Of the total chlorine supply, 20.6 % moved from lower terminal locations (laminae 0–6 in this case) to the newer growth. It may be inferred that this 20.6 % was retranslocated. The graph further shows that retranslocation also occurred from the laminae 7–11 between 40 and 55 days.

Similar calculations from figure 3 (adequate-chlorine plants) indicate that the chlorine content of the older growth decreased by 22.1 % of the total supply between 31 and 55 days. Of the 22.1 %, 11.0 % was lost from non-terminal locations. Eleven and one-tenth per cent moved from terminal locations (laminae 0–6 and laminae 7–11) by retranslocation. Here, the loss from older fractions of 22.1 % of the total chlorine supply may be compared with the 25.9 % moving into new growth after the 2nd harvest. The adequate-chlorine plants increased in dry weight from 21 to 109 g per culture between the 2nd and 4th harvests.

Comparing the retranslocation figures of 20.6 % and 11.1 % in deficient- and adequate-chlorine plants, respectively, it may be observed that a larger proportion of the labeled chlorine supply was retranslocated within the deficient plants. In absolute terms, however, the larger amount of chlorine was retranslocated within the plants having an adequate supply, i.e., 7.9 μg atoms in the deficient and 30.8 μg atoms in those adequately supplied.

The percentage of the total labeled chlorine in roots decreased following its complete absorption from the culture medium. During the last interval, the percentage in roots again increased. The redistribution patterns obtained during the 31- to 55-day interval suggests that labeled chlorine first moved out of the roots and subsequently returned to areas of younger cell development by a net retranslocation from the shoot.

It is of interest that the plants receiving 38.5 μg atoms of chlorine began showing typical chlorine deficiency symptoms within a few days after the 31-day harvest. Nevertheless, these plants subsequently showed substantially the same retranslocation pattern as did the plants adequately supplied with chlorine.

As pointed out by Williams (13), during plant ontogeny there are integrated facets of redistribution, that resulting from normal metabolic activity within and among organs associated with "adolescent" growth and maturity, and that conditioned by senescent changes. In the present studies, there was no evident period of significant average dry weight decline indicative of internal disorganization of senescence

within organ fractions. Retranslocation of chloride began in "adolescence" and continued into maturity of organs.

In tables I and II the concentrations of labeled chlorine in the various fractions of the adequate-chlorine plants are expressed as μg atoms per gram dry weight. Measurements of the concentrations of total chlorine in some of the larger fractions were made by a modification of the Conway microdiffusion method (7). These measurements showed that the amount of contamination by non-labeled chlorine was insignificant when compared with the 277 μg atoms of labeled chlorine already present. The more sensitive radioactive chlorine determination was therefore taken as a measure of the total chlorine concentration.

In all cases, decreases of concentration with time are indicated. Consideration of comparable tables and figures indicates that retranslocation occurred while there was a concurrent decrease of concentration of chlorine within the plant and its fractions. It should therefore be emphasized that comparisons among tissue fractions of concentration changes with time may not be indicative of retranslocation of chlorine but rather may be related to dilution through growth. For example, from figure 3 and table I, comparison of data for laminae 7–11, at 31 days with those at 40 days, showed a total accretion of chlorine at the expense of older tissues, indicative of retranslocation, while the corresponding chlorine concentration for laminae 7–11 decreased.

From the foregoing data, it is clear that chlorine retranslocation, as defined in this paper, occurred in tomato plants. This retranslocation was from regions of higher concentration to those of lower concentration, and was concurrent with a decrease in chlorine concentration in all parts of the plant. After meristematic development, even those parts of the plant which were receiving the retranslocated chlorine were decreasing in their chlorine concentration, because the growth rate outstripped the chlorine retranslocation rate in absence of a continuing external supply.

TABLE I

LABELED CHLORINE CONCENTRATIONS IN SUBDIVIDED PLANT FRACTIONS

PLANT FRACTION		TIME IN DAYS				
		11	19	31	40	55
		Cl conc μg atoms/g				
Laminae	15–20	...	...	...	...	4.6
Stems and petioles	15–20	...	...	...	...	4.5
Laminae	12–14	...	...	...	10.5	3.3
Stems and petioles	12–14	...	...	...	11.9	2.7
Axillary growth	...	...	...	13.0	6.5	2.8
Laminae	7–11	...	...	12.2	6.6	2.3
Stems and petioles	7–11	...	...	14.4	6.0	1.2
Laminae	0–6	...	86.0	16.2	9.4	6.4
Stems and petioles	0–6	...	227.0	18.1	5.2	2.0
Roots	...	...	108.0	7.2	2.5	2.2
Thinnings	...	88.5	...	...	...	...

Chlorine supply was adequate for maximal plant growth. Total Cl supply was 277 μg atoms of chloride as KCl. Chlorine concentrations are expressed on the dry weight basis. Data from same plant parts are represented in fig 3. Compare table II and fig 3.

TABLE II

LABELED CHLORINE CONCENTRATIONS IN UNSUBDIVIDED PLANT FRACTIONS

PLANT FRACTION		TIME IN DAYS				
		11	19	31	40	55
		Cl conc μg atoms/g				
Axis and laminae	15–20	...	...	...	...	5.2
Axis and laminae	12–14	...	...	...	10.7	3.3
Axillary growth	...	...	...	11.9	7.3	3.0
Axis and laminae	7–11	...	...	11.9	7.7	2.0
Axis and laminae	0–6	...	...	14.0	7.5	3.0
Roots	...	...	...	7.4	3.3	2.4
Total plant	...	...	114.0	...	...	...
Thinnings	...	88.5	...	...	...	...

Chlorine supply was adequate for maximal plant growth. Total Cl supply was 277 μg atoms of chloride as KCl. Chlorine concentrations are expressed on the dry weight basis. Data from same plant parts are represented in fig 5. Compare table I and fig 5.

It appears that retranslocation occurs in the general direction of decreasing concentration between organs except as modified by possible concurrent local metabolic accumulation or transpiration. The same trend may be assumed between and within cells and tissues of an organ. Though these data do not indicate the direction of smaller (e.g., intracellular) movement, the cell vacuole might be regarded as a "terminal location" so that movement of a substance out of a vacuole would be regarded as retranslocation. Probably major and rapid retranslocation is primarily associated with diffusion and streaming tendencies within the plant. From the concentration changes among organs and parts thereof, it may be inferred that some component mechanisms of the over-all process of movement of a substance are reversible; metabolic accumulation in particular is unidirectional under natural conditions. During any interval of time, distributions are the net result of the several modes of movement. This view is in general accord with data of other workers and theoretical schemes of movement of materials between the organism and its supply source and within the plant (3, 4, 5, 6).

With movements of materials in plants, it is visualized that for the systems concerned (e.g., a cell system comprising two solution phases with protoplasm interposed, or an integrated root or plant system with similar structural relations) substances move as a net result inwardly toward a salt depot by any of the modes of migration or accumulation. Material may be accumulated in vacuoles or stele. Streamings aid in rapid movement to distal regions of the plant where these solutions may become salt sources for accumulation by other member systems. With continued growth and development, solute gradients between protoplasm and its bounding solution phases (and within protoplasm itself) may be modified from the steady state previously approached such that salt,

if mobile, may move in accord with the changing physico-chemical conditions thus established. Subsequent translocation can therefore be visualized as movement occurring from previous salt depots (or terminal locations) within the plant to terminal locations where concentration levels attained depend on the relative effects of net rates of water and solute movement to the depot, transpirational solution concentration, metabolic accumulation, and growth enlargement. The redistribution of salt is progressive during the ontogeny of the plant to younger and newly developing cells and organs. The extent of retranslocation is probably characteristic of the element under consideration due to interrelationships between this element and the protoplasm, and other elements in the plant. Furthermore, the supply of the substance and the environmental conditions may be expected to alter internal processes, including accumulation and retranslocation characteristic of the plant species.

Summary

A definition of "retranslocation" was established. Experiments were performed to ascertain the extent to which chloride was translocated and retranslocated in tomato and sugar beet plants.

Radiochloride was applied overnight to the leaves of tomato and sugar beet plants. The plants were harvested one and 8 days after termination of such application. Radioautographs showed that some of the applied radiochloride reached all parts of the plants. More radiochlorine had been translocated from the application site 8 days after application than had been translocated one day after application.

Known amounts of radioactive chloride were supplied to the roots of tomato plants in culture solutions at two levels—a deficient level and an adequate level. Plants were harvested at different times and the plant parts were assayed for radiochlorine, such assays being expressed as percentages of the total applied labeled chloride and as concentrations in μg atoms per gram dry weight of tissue. It was shown that some younger plant structures obtained radiochlorine at the expense of the older leaves. This demonstrated retranslocation of chlorine as defined. Retranslocation was from regions of high chlorine concentration to those of low concentration, and was concurrent with a decrease in chlorine concentration in all plant parts.

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PATHWAYS OF OXIDATION IN CELL-FREE POTATO FRACTIONS. II. PROPERTIES OF THE SOLUBLE PYRIDINE NUCLEOTIDE OXIDASE SYSTEM[1,2]

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In the previous paper (11), the oxidation of reduced diphosphopyridine nucleotide (DPNH) by isolated potato tuber mitochondria was examined. It was also shown that the supernatant fraction rapidly attacks DPNH by a reaction that does not involve molecular oxygen. More recently, it has been shown that the inclusion of cysteine in the isolating medium greatly enhances the ability of potato mitochondria to oxidize organic acids (10, 23). Accordingly, it was of interest to know whether the presence of cysteine has any effect on the reaction between the supernatant fraction and DPNH. The present study indicates that the cysteine-containing fraction does not catalyze the anaerobic DPNH attack, but it can, on the other hand, oxidize DPNH very rapidly by a reaction that requires oxygen. Thus, the soluble fraction can catalyze either an aerobic or an anaerobic reaction with DPNH, depending on whether or not cysteine is included in the grinding medium. This paper describes the properties of the oxidase system, and evidence that ascorbic acid is involved in the reaction is presented.

MATERIALS AND METHODS

Potato tubers (*Solanum tuberosum*, var. Katahdin) were shipped from Maine and stored at 10° C. Fifty grams of peeled tissue were sliced, passed through a meat-grinder, and then ground in a glass mortar with sand. The 30 ml of grinding medium was 0.5 M in sucrose, 0.05 M in phosphate buffer (pH 7.0), and 0.001 M in $MgSO_4$; when cysteine was included, its final concentration was 0.01 M. After filtering through cheesecloth, the homogenate was centrifuged at 2,000 × G for 5 minutes (Servall SS-1) to sediment the large particles. In most experiments the supernatant fraction was then spun at 100,000 × G for 1 hour in a refrigerated Spinco ultracentrifuge (Model L) and the soluble fraction decanted for use as the enzyme solution. When the intracellular localization was examined, the homogenate was centrifuged successively at 20,000 × G for 30 minutes and 100,000 × G for 1 hour; the residue at each stage was resuspended in grinding medium and recentrifuged to give washed mitochondrial and microsomal fractions respectively. Throughout all the above operations, the temperatures of the homogenate and fractions were maintained close to 0° C. The final soluble fraction was frequently frozen and stored at – 20° C for later use.

The oxidation of reduced pyridine nucleotides (PNH) was followed by measuring the decrease in optical density (OD) at 340 mμ with a Beckman DU spectrophotometer. Reactions were performed at room temperature in 1-cm cuvettes, to which the enzyme solution was added with a hypodermic syringe at zero time. When reactions were carried out in the presence of a gas other than air, a vaccine-stoppered cuvette was flushed with the appropriate gas mixture. To obtain strictly anaerobic conditions, the special cell of Lazarow and Cooperstein (14) was employed; the enzyme solution was kept in the side-arm during a 10-minute flushing with nitrogen. All reagents, coenzymes and gases were obtained from commercial sources. Reaction rates were calculated from the earliest linear portions of the time course curves; rates are expressed as OD units per minute, or micromoles PNH per minute per gram of initial tissue.

[1] Received May 16, 1957.

[2] This work was supported in part by a grant from the National Science Foundation.

RESULTS

GENERAL PROPERTIES OF REACTION WHEN HOMOGENATE CONTAINS CYSTEINE: The addition of a small volume of cysteine-containing homogenate to a solution of DPNH is followed by a rapid decrease in the optical density at 340 mμ; reaction rates were of the order of 5×10^{-2} micromoles DPNH/minute × g fresh weight. This activity is roughly 10 times greater, on a fresh weight basis, than that associated with the heavy particle or mitochondrial fraction (11). More than 90 % of the activity was recovered in the final supernatant fraction, after centrifuging for 1 hour at 100,000 × G; this soluble fraction was used in all subsequent experiments. The washed microsomal pellet showed essentially no reaction with DPNH. The rapid reaction between DPNH and the soluble fraction is probably enzymatic in nature, since boiling destroys all the activity. The reaction rate remains essentially constant throughout and falls off in velocity only when the substrate is virtually completely oxidized (fig 1, curve A). The soluble system is relatively stable at low temperatures, the reaction rate declining only 35 % after storage for one week at – 20° C; many of the following experiments were carried out with previously frozen preparations. Reduced triphosphopyridine nucleotide (TPNH) can also be attacked by the soluble fraction and the ratio of rates of oxidation of DPNH/TPNH is roughly 1.5.

As shown in figure 1, curve A, the reaction with DPNH does not proceed under anaerobic conditions, but it goes rapidly to completion after the addition of oxygen, indicating that a true DPNH oxidase system is involved. The reaction thus differs sharply from the attack on DPNH which is catalyzed by a soluble fraction prepared in the absence of cysteine; in this case, the attack proceeds rapidly in the absence of oxygen (fig 1, curve B). Some indication of the relative oxygen affinity of the terminal enzyme in the DPNH oxidase system was obtained by carrying out the reaction in solutions flushed with various gas mix-

tures. The rates of reaction in 5, 20, and 100 % oxygen were 22, 29, and 50 OD units/minute respectively. The fact that the rate in 20 % O_2 is only 60 % of the rate in 100 % O_2 suggests a relatively low oxygen affinity, comparable to that of flavin or copper-containing oxidases.

In order to demonstrate that DPN is the reaction product, an attempt was made to regenerate the DPNH. After the initial DPNH had been completely oxidized at pH 7, 1.5 ml of the reaction mixture was transferred to a cuvette containing TRIS buffer, pH 9; the oxidase is 90 % inhibited at the higher pH. Malate was then added as a hydrogen donor, since the endogenous malic dehydrogenase can catalyze the reduction of DPN at pH 9. The addition of malate resulted in an increase in OD at 340 mμ, indicating that DPNH was regenerated (fig 2). The theoretical amount that could be regenerated was calculated from the equilibrium constant, K^1 = (oxalacetate) (DPNH)/(malate) (DPN), and the concentration of the reactants. The constant, K^1, is markedly dependent on pH, and its value was calculated from the expression of Burton and Wilson (5), $K^1 = K_c \times$ antilog pH, using their mean value of 7.5×10^{-13} for K_c. The concentration of malate added is known (0.02 M), the DPN concentration is assumed to be equal to the original DPNH (from initial OD), and the amount of oxalacetate is assumed to be negligible. In order to prevent the breakdown of DPN by DPNase, 0.1 M nicotinamide was included in the original reaction mixture. Under these conditions, DPNH regeneration was 90 % of the theoretical in one experiment (fig 2). Clearly, DPN is the major product of the DPNH oxidase reaction.

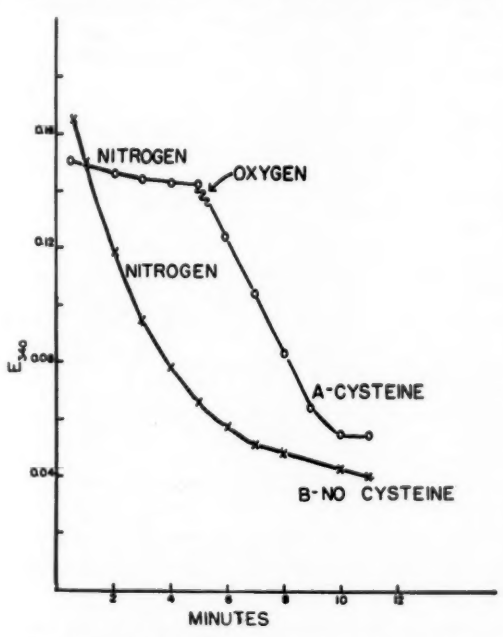

FIG. 1. The effect of anaerobic conditions on the reaction between DPNH and potato soluble fractions (A) with and (B) without cysteine. Cells contained DPNH, 0.25 M sucrose, 0.025 M phosphate buffer (pH 7). 0.3 ml enzyme added to side-arm of special cell (14) and tipped in after flushing with N_2 for 10 min. Final concentration of cysteine in (A) was 0.001 M.

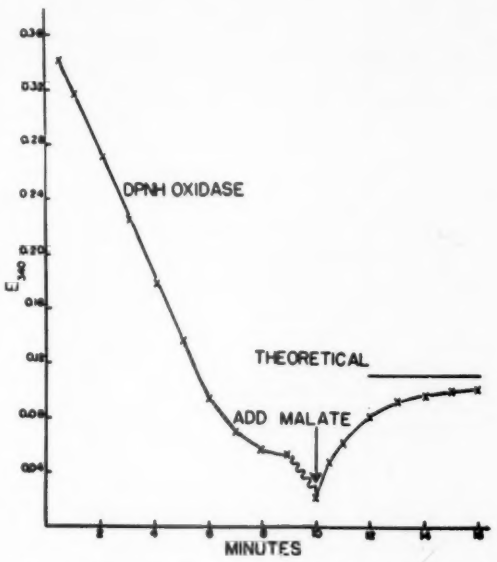

FIG. 2. Demonstration that DPN is a reaction product. Initially, cuvette contained 5.7×10^{-5} M DPNH, 0.25 M sucrose, 0.025 M phosphate buffer (pH 7), 0.1 M nicotinamide, and 0.3 ml enzyme. After 9 min, 1.5 ml reaction solution transferred to cuvette containing 1 ml of 0.15 M TRIS buffer (pH 9.3); malate added to give 0.02 M in 3 ml.

EFFECTS OF INHIBITORS: Table I shows the effects of some compounds which inhibit the activities of metal-containing enzymes. The fact that cyanide, at a concentration of 10^{-3} M, can essentially completely block the soluble DPNH oxidase system suggests that a metal-containing oxidase is involved. The oxidation of TPNH can also be prevented by cyanide. Nevertheless, the cyanide-sensitivity of this oxidase is considerably less than that of the mitochondrial cytochrome c oxidase, which is inhibited 95 % by 10^{-4} M cyanide (11). The fact that the copper-binding agent diethyl dithiocarbamate (DIECA) is an even more powerful inhibitor than cyanide suggests that the soluble oxidase contains copper. Phenylthiourea (PTU), which inhibits isolated polyphenol oxidase 50 % at 2.5×10^{-7} M (9), only inhibited the DPNH oxidase 29 % when used at 10^{-3} M. A further piece of evidence against the participation of polyphenol oxidase is the lack of carbon monoxide inhibition. The results suggest that the terminal oxidase may be a copper enzyme such as ascorbic acid oxidase.

A variety of inhibitors were tested in an effort to

TABLE I

EFFECTS OF SOME INHIBITORS OF (1) METAL-CONTAINING ENZYMES AND (2) SULFHYDRYL GROUPS ON THE DPNH OXIDASE SYSTEM

	INHIBITOR	PERCENT INHIBITION *	
(1)	0.0001 M NaCN	57	(2)
	0.001 M NaCN	90	(4)
	0.0001 M Diethyldithiocarbamate	82	(2)
	0.001 M Diethyldithiocarbamate	91	(2)
	0.001 M Ethylenediaminetetra acetate	12	(1)
	0.01 M Ethylenediaminetetra acetate	77	(2)
	0.001 M Phenylthiourea	29	(1)
	95/5 CO/O_2	0	(1)
(2)	0.001 M Iodoacetate	0	(1)
	0.0001 M Phenylmercuric acetate	39	(1)
	0.001 M Phenylmercuric acetate	100	(2)
	0.0001 M Parachloromercuri benzoate	0	(1)
	0.001 M Parachloromercuri benzoate	100	(1)

* Relative to initial rate of oxidation in control, which contained DPNH (3×10^{-5} M), sucrose-phosphate buffer (pH 7.0), and potato soluble fraction with cysteine. Values represent averages of number of experiments indicated in brackets.

block the hydrogen transport at some point between DPNH and the terminal oxidase. The following compounds had essentially no inhibitory effect: 5×10^{-4} M atabrine, 1.7 μg/ml Antimycin A, 1×10^{-3} M amytal, 1×10^{-4} M 2,4-dinitrophenol (DNP), 1×10^{-4} M $ZnSO_4$. It is not surprising that inhibitors which act on various mitochondrial oxidations are without effect on the soluble fraction. Neither the DNP-sensitive quinone reductase (27) nor glutathione reductase, which is inhibited by Zn ions (26), seems to be involved. Evidence was sought for a requirement of an essential sulfhydryl (-SH) group (table I). The complete inhibition by the two mercurials strongly suggests such a requirement. The apparent insensitivity of certain -SH inhibitors, e.g., iodoacetate, may be correlated with the presence of 10^{-3} M cysteine, which is introduced into the reaction mixture by the soluble fraction itself.

EXPERIMENTS WITH DIALYZED FRACTIONS: Dialysis of the soluble fraction against TRIS (hydroxymethyl-aminomethane, 0.05 M) buffer, pH 7, for 24 hours at 4° C completely eliminates its ability to oxidize DPNH and TPNH (fig 3). The activity can be partially restored by adding an equal aliquot of a boiled fraction, which alone shows no activity (fig 3). This reconstructed system oxidized DPNH at roughly half the rate of the undialyzed control (kept 24 hours at 4° C); the enzyme is diluted 30 % during the dialysis and this would account for some of the difference. It is clear that the original DPNH oxidase system requires the participation of both a dialyzable, heat-stable factor and non-dialyzable, heat-labile components. Accordingly, a variety of compounds were added to the dialyzed preparation in an attempt to reactivate the DPNH oxidase. The following inorganic compounds had essentially no effect: 3×10^{-3} M $NaNO_3$, 10^{-3} M $MgSO_4$, 10^{-3} M $MnSO_4$, 3×10^{-4} M H_2O_2; the absence of any effect with the last two suggests that the original reaction does not involve a DPNH peroxidase. The addition of cysteine, cystine, oxidized and reduced glutathione, at concentrations of 10^{-2} and 10^{-3} M, activated little or no DPNH oxidation. Apparently the soluble fraction lacks enzymes which can catalyze the transfer of hydrogen from DPNH to either cystine or oxidized glutathione.

In the presence of a suitable hydrogen (or electron) acceptor, the dialyzed fraction can readily oxidize DPNH. For example, the inclusion of 10^{-3} M pyruvate in the reaction mixture permits the oxidation of DPNH at a rate comparable to that measured with the undialyzed fraction. This finding confirms the report that potato extracts contain an active lactic dehydrogenase (7). Since this reaction is not affected by 10^{-3} M cyanide, it cannot be invoked to explain the original activity. DPNH is also rapidly oxidized by the dialyzed fraction in the presence of ferricyanide or 2,6-dichlorophenol-indophenol (fig 3); there was no reaction with methylene blue. The rate of the diaphorase reaction was similar to that of the DPNH oxidase in the undialyzed supernatant fraction. Added mammalian cytochrome c, 2 to 6×10^{-5} M, had no effect (fig 3), indicating that this soluble prepara-

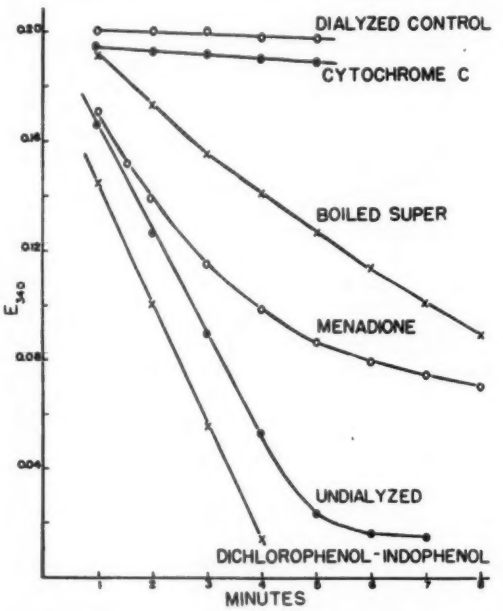

FIG. 3. The effect of dialysis on the DPNH oxidase reaction, and effects of additions to the dialyzed fraction. All cuvettes contained DPNH, 0.25 M sucrose, 0.025 M phosphate buffer (pH 7) and 0.3 ml dialyzed fraction (undialyzed where indicated). Final concentrations of additions: 2×10^{-5} M cytochrome c, 10^{-4} M menadione, 10^{-4} M 2,6-dichlorophenol-indophenol, and 0.3 ml boiled, undialyzed supernatant in 3.0 ml.

tion does not possess a DPNH-cytochrome c reductase system.

Menadione (2-methyl, 1,4-naphthoquinone) can serve as an effective hydrogen acceptor (fig 3). There is no reaction between DPNH and menadione either in the absence of the dialyzed fraction or in the presence of boiled enzyme, indicating that a DPNH-menadione reductase system is present. However, this reaction is inhibited only 20 % by 10^{-4} M dinitrophenol, in contrast to the DNP-sensitive quinone reductase described earlier (27). The DPNH-menadione reaction is completely prevented by 10^{-2} M cysteine, probably due to the formation of a product between cysteine and menadione (28), and this fact argues strongly against the participation of quinones as intermediate carriers in the DPNH oxidase system of the cysteine-containing supernatant.

Ascorbate-Activated Reaction: The effects of inhibitors on the DPNH oxidase system suggested that ascorbic acid oxidase might be involved in the reaction. This possibility is supported by the fact that addition of a suitable concentration of ascorbic acid to the dialyzed supernatant results in a rapid oxidation of DPNH (fig 4). The optimum ascorbate concentration is from 10^{-2} to 10^{-3} M, and the maximum oxidation rates were roughly one-half those measured with an equivalent amount of the original undialyzed fraction, i.e., around 2×10^{-2} micromoles DPNH/min × g. In one experiment, the rate of ascorbate-activated DPNH oxidation, corrected for dilution of enzyme during dialysis, was 73 % of the original rate. This suggests strongly that much, if not all, of the DPNH oxidase requires the participation of ascorbic acid. Ascorbate was also able to activate the oxidation of TPNH by the dialyzed fraction. It is of considerable interest that dehydroascorbic acid is unable to activate DPNH oxidation.

The properties of the ascorbate-activated reaction were studied in order to permit a direct comparison with the original DPNH oxidase. There is no reaction under anaerobic conditions, and the participation of an oxidase is clearly indicated by the fact that it proceeds rapidly when oxygen is added. As with the undialyzed preparation, the rate was markedly increased when 100 % O_2 was substituted for air in the reaction mixture. Using malate as a hydrogen donor, it was possible to show that DPN is a product of the reaction. The system is not inactivated by freezing for several days. The following inhibitions of the ascorbate-activated reaction were measured: 10^{-3} M cyanide, 44 %; 10^{-3} M DIECA, 75 %; 10^{-4} M PTU, 9 %; 10^{-4} M phenylmercuricacetate, 100 %; 10^{-4} M parachloromercuribenzoate (PCMBA), 100 %. These results suggest that both a copper-containing enzyme (oxidase) and an essential -SH group are involved in the ascorbate-activated reaction.

Reaction in Absence of Cysteine: It was reported earlier that the 10,000 × G supernatant fraction from a sucrose-phosphate homogenate also attacks DPNH rapidly, as indicated by the decrease in OD at 340 mμ (11). Subsequent experiments have

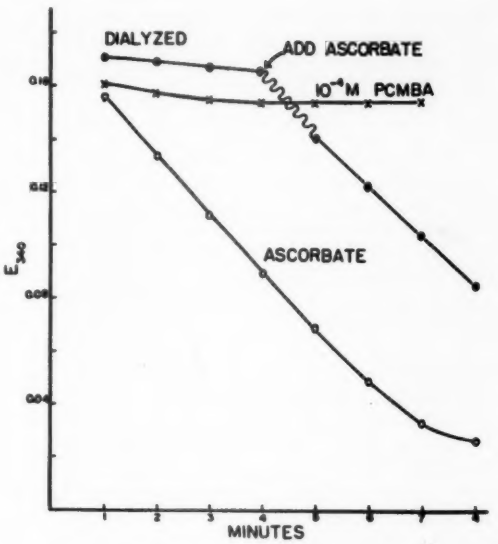

Fig. 4. Activation of the DPNH oxidase reaction in the dialyzed fraction by ascorbate, and its inhibition by parachloromercuribenzoate (PCMBA). All cuvettes contained DPNH, 0.25 M sucrose, 0.025 M phosphate buffer (pH 7), and 0.3 ml enzyme. 0.001 M ascorbate present initially or added after 4 min (where indicated); PCMBA, 10^{-4} M; final volume, 3.0 ml.

shown that this reaction is catalyzed by soluble components which are not sedimented at 100,000 × G (1 hour). The rate of DPNH attack by this system is in the range 3 to 4×10^{-2} micromoles DPNH/min × g fresh weight, or roughly half that of the DPNH oxidase described above. Unlike the DPNH oxidase reaction, the time course curve does not remain linear (fig 1). The system is completely inactivated by boiling, but freezing and storage for several days at – 20° C has no effect. Dialysis removes all the activity, but the control kept for 24 hours at 4° C also shows a greatly diminished activity.

The reaction between DPNH and this fraction proceeds under anaerobic conditions (fig 1, curve B), indicating that it does not involve an oxidase. This conclusion is supported by the fact that the reaction is relatively insensitive to typical oxidase inhibitors. For example, the following inhibitions were measured: 5×10^{-4} M cyanide, 21 %; 10^{-3} M cyanide, 32 %; 10^{-4} M DIECA, 6 %; 10^{-3} M DIECA, 13 %. A comparison of these results with earlier data (table I) emphasizes the difference between this reaction and the DPNH oxidase of the cysteine-containing supernatant fraction. The reaction is also relatively insensitive to -SH inhibitors: 10^{-4} M PCMBA had essentially no effect, and 10^{-3} M phenylmercuricacetate inhibited roughly 60 %. Cysteine (0.01 M) in the reaction mixture does not block the anaerobic DPNH attack. The quinone and glutathione reductase inhibitors, 10^{-4} M DNP and 10^{-4} M $ZnSO_4$, did not in-

hibit. The reaction also proceeded normally when DPN was included to give a DPN/DPNH ratio of 2/1; this argues against a modification reaction of the type described by Rafter et al (22).

Discussion

All of the evidence suggests that the terminal reaction with oxygen in the soluble DPNH oxidase system is mediated by ascorbic acid oxidase. The linear reaction rate observed here is characteristic of ascorbic acid oxidation (12). The oxygen affinity of the DPNH oxidase is similar to that of purified ascorbic acid oxidase (25). There is a close correspondence in inhibitor sensitivities, for it is well known that ascorbic oxidation both by plant juices (24) and by the purified oxidase (8) is effectively blocked by cyanide and DIECA. Further evidence comes from the finding that ascorbic acid can activate the dialyzed supernatant fraction to catalyze a DPNH oxidation that is both qualitatively and quantitatively similar to the reaction in the undialyzed, cysteine-containing fraction. Assuming that the amount of ascorbic acid in potato tubers is 15 mg/100 g (3, 4), the concentration in the homogenates would be between 10^{-3} and 10^{-4} M; a similar concentration range is effective in activating the dialyzed supernatant. The oxidation rate of 5×10^{-2} micromoles DPNH/min × g fresh weight would correspond to an oxygen consumption of 34 μl/hr × g.

In some plant extracts, the oxidation of reduced pyridine nucleotides (PNH) can be coupled to ascorbic acid oxidase by intermediate sulfhydryl carriers, such as cysteine and glutathione, which reduce dehydroascorbic acid. Such a mechanism does not seem to be involved in the potato extract; neither cystine nor oxidized glutathione served as an effective hydrogen acceptor for DPNH. In general, glutathione reductase is specific for TPNH (17, 26). The finding that ascorbic acid alone, and not dehydroascorbic acid, activates the DPNH oxidase also argues against this type of coupling mechanism.

Matthews (19) first noted that PNH oxidation by plant extracts may be linked to ascorbic acid by a reaction which does not utilize dehydroascorbic acid. Several workers, (2, 13, 20) have now shown that the oxidation of ascorbate by its oxidase generates a labile product (monodehydroascorbate?) which can accept hydrogen from PNH. Although the details of this reaction have not been worked out, there is evidence that it involves a flavoprotein (13) and a component that is very sensitive to -SH reagents (2); the latter factor may be concerned with the reduction of flavin by PNH (21). All of the evidence obtained with the potato extracts—the participation of ascorbic acid oxidase, the activation by ascorbate alone, the presence of an active diaphorase (flavoprotein), the sensitivity to -SH inhibitors—suggests that this type of reaction is involved in the DPNH oxidase system. The reaction with TPNH was not investigated in detail, but it is probably oxidized by a similar pathway. Although TPNH could not be substituted for DPNH in the cucumber oxidase system (2), other workers (13, 19, 20) have indicated that TPNH oxidation can be directly coupled to the ascorbic acid oxidase system. The TPNH oxidase of Conn et al (6), which involves a peroxidase and is inhibited by ascorbate, is probably not responsible for the reaction studied here, although its participation has not been ruled out.

The demonstration that ascorbic acid can act as a component of an extracted DPNH oxidase does not establish its role as a respiratory carrier in vivo. Indeed, at least 70 % of the respiration of freshly cut potato slices is mediated by cytochrome oxidase (15, 25), and the remaining cyanide-insensitive fraction probably does not involve ascorbic acid oxidase. The indirect evidence for a respiratory role based on alterations in the levels of glutathione and ascorbate in potato tubers (1) is not conclusive on this point. Thus, it is most likely that the active DPNH oxidase examined here is an "isolation artifact" which is not physiologically important for respiration. This conclusion may also obtain for the PNH-ascorbic acid oxidase systems which have been demonstrated in various soluble (16, 18) and particulate (29) plant fractions.

The rapid DPNH attack by the homogenate which does not contain cysteine involves an entirely different type of reaction; it proceeds anaerobically and is much less sensitive to -SH inhibitors. The evidence suggests that the anaerobic and aerobic reactions with DPNH are mutually exclusive, since the conditions which favor one prevent the other. This could be explained as follows: in the absence of cysteine, endogenous ascorbate is all oxidized to dehydroascorbic acid and no DPNH oxidase can be demonstrated; on the other hand, a variety of oxidized compounds, e.g., quinones, are formed, and these can serve as hydrogen acceptors under anaerobic conditions. In the presence of cysteine, ascorbate is kept reduced and it can participate in the DPNH oxidase system; no suitable hydrogen acceptors for the anaerobic reaction are formed. It may be that such an effect of cysteine, i.e., prevention of the formation of potentially injurious oxidation products, accounts for its protective action during the isolation of potato mitochondria.

Summary

The soluble fraction prepared from a sucrose-phosphate-cysteine homogenate of potato tuber tissue catalyzes the rapid oxidation of DPNH and TPNH. The reaction requires molecular oxygen and can be inhibited by cyanide and diethyldithiocarbamate; it is also blocked by certain -SH group inhibitors. Dialysis of the fraction removes all activity. The dialyzed fraction can oxidize DPNH in the presence of added ascorbate or a suitable hydrogen acceptor, such as pyruvate, 2,6-dichlorophenol-indophenol, ferricyanide, or menadione. The ascorbate-activated reaction is qualitatively similar to that catalyzed by the undialyzed fraction. It is concluded that the original DPNH (TPNH) oxidase reaction involves both ascorbic acid and a step that is sensitive to -SH inhibitors.

The soluble fraction prepared in the absence of cysteine also attacks DPNH rapidly, but this reaction does not require oxygen and is less sensitive to the inhibitors tested. It is suggested that the presence of cysteine in the grinding medium not only permits the soluble DPNH oxidase to operate, but also prevents the formation of components required for the anaerobic DPNH attack.

The author gratefully acknowledges the able assistance of Mr. Darrell W. Haas and Mr. Donald J. Niederpruem.

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ANTHOCYANINS IN BRACTS OF EUPHORBIA PULCHERRIMA AS REVEALED BY PAPER CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC METHODS[1]

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Lawrence et al (4) identified the red pigment in bracts of poinsettia as an anthocyanin. The methods employed served to identify the principal anthocyanidin (cyanidin), but not the sugar moiety. Blank (2), Robinson and Robinson (5, 6, 7), and Scott-Moncrieff (8) reported that variation in the sugar moiety of the anthocyanin pigment affected color. They found that monoglycosides were redder than the corresponding pentose glycosides, while 3-biosides and 3,5-diglycosides were bluer. The purpose of the present investigation was to isolate and identify by paper chromatographic and spectrophotometric methods the anthocyanidin as well as the sugar moiety of the anthocyanin in bracts of *Euphorbia pulcherrima*.

Methods and Materials

Bracts from plants of diploid Ruth Ecke (standard red) poinsettia, a seedling of tetraploid Ruth Ecke (dark red), and a cross between Ruth Ecke and Ecke's White (scarlet red) were used. The bracts were dried at 70° C in a forced-draft oven and then ground separately in a Wiley mill to pass a 20-mesh screen. A 4-g sample of the ground tissue was extracted with three successive 100-ml portions of cold 1 % hydrochloric acid by macerating in a Waring blendor for five minutes. The combined extracts were filtered, and the anthocyanin was precipitated with neutral lead acetate. The supernatant was separated by filtration and the lead salt was washed with distilled water and then dried in vacuo in the dark at room temperature. Ten ml of a methanolic solvent containing 2 % hydrochloric acid was added to a 300-mg sample of the lead salt and held in the dark at 5° C for 24 hours. The lead chloride precipitate was removed by centrifugation and the clear supernatant solution containing the anthocyanin was subjected to paper chromatographic separation. Chromatograms were prepared by streaking the red solution in a band approximately 1 cm wide across the narrow width of Whatman No. 3 MM paper (18 by 22 inches). The papers were irrigated in a chromatographic cabinet by the descending method with a solvent of 1-butanol : acetic acid : water (4 : 1 : 5 v/v). The separated anthocyanin bands were cut out and eluted with 50 % methanol and the eluant was concentrated under reduced pressure. The anthocyanin was further puri-

[1] Received June 18, 1957.

Table I

R_f Values and Wave Lengths of Maximum Absorption for the Anthocyanins in Bracts of Poinsettia

Compounds	R_f value in				λ max. in ethanol containing .01 *N* hydrochloric acid (mμ)
	Acetic acid (5 %)	1-Butanol : acetic acid : water (4 : 1 : 5 v/v)	Phenol : water (73 : 27 w/w)	m-Cresol : acetic acid : water (50 : 2 : 48 v/v)	
Poinsettia anthocyanins:					
Band 1 from —					
Ruth Ecke(diploid)	.26	.32	.51	.43	538
Ruth Ecke (tetraploid)	.25	.33	.51	.42	538
Ruth Ecke × Ecke's White	.26	.32	.51	.44	538
Band 2 from —					
Ruth Ecke (diploid)	.39	.28	.49	.31	539
Ruth Ecke (tetraploid)	.38	.29	.47	.32	539
Ruth Ecke × Ecke's White	.39	.28	.49	.32	539
Band 3 from —					
Ruth Ecke (diploid)	.36	.52	.78	.62	518
Ruth Ecke (tetraploid)	.36	.51	.78	.64	518
Ruth Ecke × Ecke's White	.36	.52	.78	.63	518
Band 4 from —					
Ruth Ecke (diploid)	.50	.44	.75	.50	518
Ruth Ecke (tetraploid)	.50	.44	.75	.50	518
Ruth Ecke × Ecke's White	.50	.44	.75	.50	518
Authentic anthocyanins:					
Idaein (cyanidin-3-galactoside)	.25	.32	.65	.50	538
Chrysanthemin (cyanidin-3-glucoside)	.27	.32	.53	.44	538
Antirrhinin (cyanidin-3-rhamnoglucoside)	.37	.28	.49	.32	540
Callistephin (pelargonidin-3-glucoside)	.34	.55	.82	.68	517
Pelargonin (pelargonidin-3,5-diglucoside)	—	.34	.68	.42	518

TABLE II

R_f VALUES AND WAVE LENGTHS OF MAXIMUM ABSORPTION FOR ANTHOCYANIDINS FROM ACID HYDROLYSIS OF THE ANTHOCYANINS IN BRACTS OF POINSETTIA

COMPOUNDS	R_f VALUE IN			λ MAX. IN ETHANOL CONTAINING .01 *N* HYDROCHLORIC ACID (mμ)
	ACETIC ACID: HYDROCHLORIC ACID: WATER (30:3:10 v/v)	M-CRESOL: 5.5 *N* HYDROCHLORIC ACID: ACETIC ACID (1:1:1 v/v)	1-BUTANOL: 2 *N* HYDROCHLORIC ACID (1:1 v/v)	
Poinsettia anthocyanidins:				
Band 1 from —				
Ruth Ecke (diploid)	.59	.75	.69	545
Ruth Ecke (tetraploid)	.60	.74	.67	545
Ruth Ecke × Ecke's White	.60	.74	.68	545
Band 2 from —				
Ruth Ecke (diploid)	.60	.74	.70	544
Ruth Ecke (tetraploid)	.60	.74	.68	544
Ruth Ecke × Ecke's White	.60	.75	.69	544
Band 3 from —				
Ruth Ecke (diploid)	.76	.80	.80	532
Ruth Ecke (tetraploid)	.76	.80	.80	532
Ruth Ecke × Ecke's White	.76	.80	.80	532
Band 4 from —				
Ruth Ecke (diploid)	.75	.81	.80	532
Ruth Ecke (tetraploid)	.75	.81	.80	532
Ruth Ecke × Ecke's White	.75	.81	.80	532
Authentic anthocyanidins:				
Delphinidin	.38	.57	.35	555
Cyanidin	.60	.74	.69	545
Pelargonidin	.74	.82	.80	530

TABLE III

R_g VALUES AND COLOR WITH ANILINE HYDROGEN PHTHLATE OF SUGARS FROM ACID HYDROLYSIS OF ANTHOCYANINS IN BRACTS OF POINSETTIA

COMPOUNDS	R_g VALUE IN			COLOR WITH ANILINE HYDROGEN PHTHALATE
	ETHYL ACETATE: PYRIDINE: WATER (8:2:1 v/v)	1-BUTANOL: ETHANOL: WATER (40:11:19 v/v)	PHENOL: WATER (73:27 w/w)	
Sugars from acid hydrolysis of anthocyanins:				
Band 1 from —				
Ruth Ecke (diploid)	.97	.98	1.00	Brown
Ruth Ecke (tetraploid)	.97	.98	.97	Brown
Ruth Ecke × Ecke's White	1.00	1.00	1.00	Brown
Band 2 from —				
Ruth Ecke (diploid)	.97; 1.95	.99; 1.76	1.00; 1.62	Brown
Ruth Ecke (tetraploid)	.97; 1.96	1.00; 1.79	1.00; 1.61	Brown
Ruth Ecke × Ecke's White	.97; 1.96	1.00; 1.78	.98; 1.60	Brown
Band 3 from —				
Ruth Ecke (diploid)	1.00	1.00	.97	Brown
Ruth Ecke (tetraploid)	.99	.98	.99	Brown
Ruth Ecke × Ecke's White	1.00	1.00	1.00	Brown
Band 4 from —				
Ruth Ecke (diploid)	1.00; 1.97	.99; 1.76	.99; 1.60	Brown
Ruth Ecke (tetraploid)	1.00; 1.97	1.00; 1.79	1.00; 1.61	Brown
Ruth Ecke × Ecke's White	1.00; 1.97	1.00; 1.78	1.00; 1.62	Brown
Authentic sugars:				
Galactose	.89	.92	1.07	Brown
Glucose	1.00	1.00	1.00	Brown
Arabinose	1.41	1.21	1.38	Red
Fucose	1.63	1.51	1.64	Brown
Lyxose	1.81	1.43	1.34	Red
Xylose	1.70	1.31	1.24	Red
Rhamnose	1.97	1.78	1.62	Brown

fied by rechromatographing as previously described but using 5 % acetic acid as the solvent.

For the determination of R_f values a suitable portion of the purified anthocyanin extract was applied with a capillary tube to Whatman No. 1 paper (7 by 22 inches) at intervals of 2.5 cm along the starting line and irrigated by the descending method with the solvents listed in table I.

A 1-ml portion of the concentrated anthocyanin eluate was hydrolyzed with 1 ml of 2 *N* hydrochloric acid by heating at 100° C under reflux for 30 minutes. The anthocyanidin fraction was extracted three times with a minimum amount of *n*-amyl alcohol and the aqueous phase was examined for sugars.

R_f values for the anthocyanidin were determined by ascending chromatography. A suitable portion of the extract was spotted on 15- by 16-inch sheets of Whatman No. 1 paper rolled into cylinders as described by Irreverre and Martin (3), and irrigated with the solvents listed in table II. Identification of the anthocyanidins was made by co-chromatography with authentic compounds.

The aqueous phase containing the sugars was deacidified by passage through a weak anion-exchange resin (Deacidite E) and then concentrated to about 0.1 ml under reduced pressure at 40° C. Aliquots of 5 to 20 μl of the concentrated sugar fraction and 5 μl of 1 % solutions of authentic sugars, respectively, were applied with capillary tubes to Whatman No. 1 paper (7 by 22 inches) at intervals of 2.5 cm along the starting line. The front edge of the paper was trimmed with pinking shears to promote faster and more uniform movement of the solvent front. The papers were irrigated by the descending method with the solvents listed in table III. The sugars were located on the air-dried chromatograms by momentarily dipping them into aniline hydrogen phthalate (1), removing the excess by blotting, and then heating them at 100° C for three minutes. Identification was made by co-chromatography with authentic compounds.

The absorption spectra of anthocyanins and anthocyanidins were determined with a Beckman DU spectrophotometer using an ethanolic solvent containing .01 *N* hydrochloric acid.

Results and Discussion

The anthocyanins extracted from the bracts from each of the poinsettia plants, when originally separated by paper chromatography resolved into two distinct red bands and one obscure orange-scarlet band. When the orange-scarlet band was examined in several solvent systems (table I) it was found to contain two components. The R_f values and wave lengths of maximum absorption for the anthocyanins are shown in table I. These data indicate that the anthocyanins in the bracts of all the poinsettia plants examined were probably the same. Those in bands one and two were cyanidin glycosides and those in bands three and four were pelargonidin glycosides.

To identify further the anthocyanins, the four pigments from the bracts from each of the poinsettia plants were subjected to acid hydrolysis, and the products were identified by paper chromatographic and spectrophotometric methods. The anthocyanidin produced from bands one and two was cyanidin and that from bands three and four pelargonidin (table II). Hydrolysis of bands one and three yielded one sugar identified as glucose and of bands two and four, two sugars identified as glucose and rhamnose (table III).

Although the positions of the individual sugars were not determined it is apparent from the data in tables I, II and III that the anthocyanins in the bracts of all the poinsettia plants examined were identical. The anthocyanins were cyanidin-3-glucoside (chrysanthemin), cyanidin-3-rhamnoglucoside (antirrhinin), pelargonidin-3-glucoside (callistephin) and pelargonidin rhamnoglucoside. The R_f values of the pelargonidin rhamnoglucoside indicate that the sugars are probably attached at the 3-position as rutinose. If the sugars were attached at the 3- and 5-positions, the R_f values for this anthocyanin probably would have been similar to those shown in table I for pelargonidin-3,5-diglucoside (pelargonin).

On the basis of the quantity of pigment in the bracts of the poinsettia plants examined, the cyanidin glycosides constituted the major components and the pelargonidin glycosides the minor ones.

Summary

The anthocyanins in the bracts from poinsettia plants of diploid Ruth Ecke (standard red), a seedling of tetraploid Ruth Ecke (dark red), and a cross between Ruth Ecke and Ecke's White (scarlet red) were isolated and identified by paper chromatographic and spectrophotometric methods. Acid hydrolysis of the anthocyanins and identification of the anthocyanidin and sugar moieties indicated that the anthocyanins in the bracts from all the poinsettia plants examined were identical. They were cyanidin-3-glucoside (chrysanthemin), cyanidin-3-rhamnoglucoside (antirrhinin), pelargonidin-3-glucoside (callistephin), and pelargonidin-3-rhamnoglucoside.

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CURVATURES AND MALFORMATIONS IN BEAN PLANTS CAUSED BY CULTURE FILTRATE OF ASPERGILLUS NIGER[1,2]

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The fungus, *Aspergillus niger,* has been demonstrated to produce a growth substance active in the Avena test (4, 16). Boysen-Jensen (5) showed that the active material was soluble in ether under slightly acid conditions and that the fungus could convert tryptophan, lysine, leucine, tyrosine, histidine and phenylalanine into growth substance. The work of Kögl and Kostermans (12) established further that the molecular weight of the growth regulator was 169, a value which closely approximates that expected for indoleacetic acid (IAA). These lines of evidence suggest that IAA is produced by *A. niger.* IAA has been clearly shown to be the growth substance in the culture filtrate of *Rhizopus suinus* (18).

As a part of a routine screening program, seeking to detect fungus products that influence the growth of higher plants, it was recently discovered that culture filtrates of a fungus isolated from soil induced in plants effects which somewhat resemble those caused by known growth substances. However, the effects are distinctly different from those caused by IAA and evidence will be presented which eliminate IAA as the causative agent. Other preliminary studies concerning the active compound will also be reported in the present paper. The fungus responsible for these effects is designated as 56-39 and has been identified as *A. niger.*

Materials and Methods

Culture Conditions: The methods used to obtain filtrates from fungi routinely isolated from soil have been described (6). The fungi were grown in 500-ml Erlenmeyer flasks containing 100 ml of corn steep-Cerelose[3] medium (Staley's corn steep liquor, 40.0 g; Cerelose, 40.0 g; $CaCO_3$, 3.5 g; $NaNO_3$, 3.0 g; K_2HPO_4, 0.5 g; $MgSO_4$, 0.25 g; deionized water, 1000 ml). The inoculated flasks were incubated on a reciprocating shaker at 28° C for 7 days. The growth in each flask was removed by filtration (Whatman No. 1) and discarded. One drop of Tween 80 was added to approximately 100 ml of culture filtrate, adjusted to pH 5.0. All filtrates were initially tested without dilution.

Treatment of Plants with Culture Filtrates: For purposes of routine screening, seedlings of corn (the single cross WF9 × 38-11) and bean (var. Black Valentine) have been used.

Corn seedlings were treated when 6 to 8 cm in height by filling the whorls with the culture filtrate on each of two alternate days. Plant heights were measured after 10 to 12 days and other effects of the treatment were noted. However, the work reported here concerns only the activity of the culture filtrate on bean plants, since little or no effect has been observed on corn.

Bean seedlings were ordinarily treated when the primary leaves had expanded to about one third of their full size and before elongation of the epicotyl had begun. This was done by placing 40 to 50 droplets of culture filtrate on one of the primary leaves on each of two alternate days. For reasons which will be made clear later, this technique was modified so that plants were treated only on the young growing point before the epicotyl began to elongate.

Results

Description of Activity of Culture Filtrate *A. niger* 56-39: The fungus in question was originally designated as 56-39, one of some 1500 culture filtrates tested for activity on higher plants. In the initial experiment four bean plants were treated on one of the simple leaves as described. Normally, the subsequent elongation of the stem follows a straight vertical line with the compound leaves produced more or less perpendicular to the elongating stem. However, at the end of 7 to 10 days two of the plants treated with the culture filtrate of 56-39 showed marked downward curvatures of the elongating stem and of the first compound leaf, as well as malformations on the stem near the junction of the first compound leaf and on the petiole of this leaf. The experiment was repeated a number of times on a large scale using a new supply of culture filtrate. In each experiment it was only possible to produce these striking effects on approximately one half of the treated plants. A variety of experiments were conducted in an attempt to induce the effects of the treatment on all of the treated plants. These included growing the plants under a variety of conditions, treating them at different ages, treating both of the simple leaves, incubating the cultures of 56-39

[1] Received July 11, 1957.

[2] Journal Paper No. 1124 of the Purdue Agricultural Experiment Station.

[3] Trade name for technical grade glucose produced by Corn Products Refining Company.

for greater lengths of time, etc. None of these methods induced response in all of the treated plants. It was finally noted that the petioles of the simple leaves of plants responding to the treatment were slightly necrotic. This suggested that some of the filtrate applied to the primary leaf was running down the petiole and coming in contact with the growing point. As precautions were taken to prevent this, the fewer were the plants that responded to the treatment. The method of treatment was then varied to include plants treated only on the growing point. When this was done, all of the plants treated with the culture filtrate responded. All future treatments were therefore made by treating the growing point by placing a small drop of the culture filtrate on both sides of the growing point before it had begun to elongate.

Figures 1 through 4 illustrate some of the effects of the culture filtrate of *A. niger* 56-39 on bean plants. In figure 1 three of the plants treated with 56-39 culture filtrate (left) are compared with control plants treated with the uninoculated culture medium (right). The *A. niger* filtrate produced a marked growth inhibition. The 1st and 2nd internodes above the primary leaves were most severely inhibited while the 3rd internode usually showed little or no reduction in growth. The growth of plants treated with the uninoculated culture medium was comparable to that of untreated controls. Curvatures and malformations may be seen on the plants in figure 2. The 2nd compound leaf (arrow) on the left plant is curved sharply downward. Such downward curvatures, especially of the 1st and 2nd compound leaves, occur on almost all plants treated with *A. niger* filtrate. Strong downward curvatures of the stem are also of frequent occurrence. The 2nd internodes (above the primary leaves) of the middle and right plants of figure 2 also illustrate stem thickenings frequently encountered on treated plants. The thickness of the 3rd internodes of these plants closely approximates the normal size. In figure 3 sections of control (center) and *A. niger* treated plants (left and right) were cut from the plants at the junction of the primary leaves and the compound leaves were removed. The abnormal stem and petiole thickenings are clearly visible. In many cases the malformations produced on plants treated with *A. niger* filtrate have consisted of irregular stem protrusions, severely twisted stems, and stems greatly enlarged in only one plane to produce a stem which is

FIG. 1. Bean plants treated on petiole and growing point with culture filtrate of *Aspergillus niger* 56-39 (left) and uninoculated corn steep-Cerelose medium (right).

FIG. 2. Bean plants treated with *A. niger* 56-39 culture filtrate. Note stem thickening on developing epicotyl and downward curvature of compound leaf (arrow).

FIG. 3. Sections of plants treated with uninoculated culture medium (two center plants) and *A. niger* 56-39 culture filtrate (left and right). Sections cut at junction of simple leaves.

FIG. 4. First trifoliolate leaves of plants treated with uninoculated culture medium (above) and *A. niger* 56-39 culture filtrate (below).

wide and relatively flat. Finally, figure 4 illustrates the effect on the 1st compound leaf when the growing point is treated with the uninoculated medium and 56-39 culture filtrate. This effect on the 1st trifoliolate leaves, however, has been found to be caused by a large number of filtrates and even occurs at times on plants treated with the uninoculated culture medium. In summary, the culture filtrate of the fungus designated as *A. niger* 56-39 causes marked curvatures and grotesque malformations on bean plants when the growing point is treated. The effect does not appear to be obtained when only the primary leaves are treated. As the culture filtrate of 56-39 was diluted the effect on the plants diminished. Slight activity was present in culture filtrates diluted to 1/20 of their original concentration but little or no effect was noted at 1/50 dilution.

It was of interest to know whether or not the culture filtrate of 56-39 would induce curvatures in the hypocotyl and elongating epicotyl of bean plants treated unilaterally. Plants treated on one side of the hypocotyl or epicotyl gave no negative curvatures while IAA gave good curvatures. Indeed, it appeared that plants treated in this manner gave slight curvatures opposite to that of IAA, i.e., positive curvatures. These results, however, were not definite and require further investigation.

Culture Studies: To determine the length of incubation required to obtain the active compound a series of flasks containing the corn steep-Cerelose medium were inoculated with *A. niger*. These were removed from the shaker at the end of 1, 2, 4, 5, 6, and 7 days and the filtrates were tested for activity on bean plants. No curvatures were produced by filtrates obtained from cultures incubated for one day. Filtrates from cultures incubated for two days gave mild but definite curvatures, while filtrates obtained from cultures incubated for four or more days gave strong curvatures.

Attempts were made to obtain the active principle by growing the fungus on a variety of media. In addition to the regular corn steep medium the following liquid media have been employed: Czapek-Dox, Raulin-Thom, Richard's, and potato-dextrose broth. The shake-flasks were incubated for one and two weeks. Active filtrates were present at the end of two weeks using all media except the Raulin-Thom medium. Good growth occurred on all media tested.

Antibiotic Activity: Culture filtrates of 56-39 were examined for antibiotic activity using the filter paper disc method of Vincent and Vincent (20). The following organisms were used in testing for antibiotic activity: *Escherichia coli, Staphylococcus aureus, Ustilago zeae, Pichia haplophila,* and *Torulopsis nitratophila*. Antibiotic zones were obtained only on plates of *E. coli* and *S. aureus*. These zones were largest on the *E. coli* plates. In a second experiment, using culture filtrate that had been stored for several weeks, no antibiotic zones were obtained but good curvatures were produced on bean plants. Since a loss in antibiotic activity was not accompanied by a loss in activity on bean plants the two factors are probably not identical. Foster and Karow (9) have presented evidence which indicates that *A. niger* produces penicillin.

Chemical and Physical Properties of the Active Substance: The crude culture filtrate of 56-39 could be reduced to dryness at 50 to 55° C. in a forced air oven without loss of activity. Crude culture filtrates were adjusted to pH 2, 5 and 9. These were autoclaved for 20 minutes, readjusted to pH 5 and tested for activity on bean plants. The activity was present in all cases, although it appeared to be diminished when autoclaved at pH 9. In addition, concentrated culture filtrates were autoclaved in 0.5 *N* NaOH and 0.5 *N* HCl. No activity was obtained in the filtrate autoclaved in 0.5 *N* NaOH but good activity was obtained in filtrates autoclaved in 0.5 *N* HCl. The active compound is, therefore, acid stable and alkali labile.

Aliquots of the crude culture filtrate were adjusted to pH 2, 5 and 9 and decolorized with activated charcoal. This treatment removed the activity from the filtrate at all pH values used. Further experiments indicated that at least 5 g of charcoal should be used to remove all of the activity from 100 ml of the culture filtrate. The active compound could be eluted from charcoal with ethyl alcohol, acetone and ethyl acetate but not by methyl alcohol or water.

The solubility properties of the active compound were studied in the following manner: solvents miscible with water were added in excess to small volumes of the concentrated aqueous filtrate in an attempt to precipitate the compound, or the crude filtrate was reduced to dryness and the residue extracted for several hours with the solvent. Solvents immiscible with water were tested by extracting the culture filtrate three times in a separatory funnel or by extracting the dried aqueous residue. Using one or more of these techniques it has been determined that the active compound produced by *A. niger* is soluble in methyl, ethyl and *n*-amyl alcohol, acetone, chloroform, benzene and ethyl acetate. It is also soluble in ethyl ether at pH 2, 5 and 9. The compound is insoluble in xylene and petroleum ether (B.R. 60 to 70° C.). Following treatment with carbon tetrachloride some activity appeared in both the soluble and insoluble fractions.

Dialysis experiments were conducted by placing 10 ml of the concentrated filtrate on the inside of a cellophane tube. This was placed overnight in a small beaker of water agitated by a stream of air bubbles. Activity was present in the liquid surrounding the dialysis tubing. The active compound is therefore dialyzable.

The ion exchange resins IRA-400 (OH) and IR-120 (H) were used in preliminary studies of the ionic nature of the active compound. Columns of IRA-400 (OH) were washed thoroughly with 10 % NaOH while columns of IR-120 (H) were washed with 12 % HCl. This was followed by deionized water to pH 5. An ether extract of the culture of *A. niger* was re-

duced to dryness and taken up in water. This extract was divided into two aliquots. The aliquot which was run through the IRA-400 (OH) resin was adjusted to pH 8 while the aliquot for the IR-120 (H) was adjusted to pH 3. The extracts were washed through the columns with deionized water until a total of ten 10-ml fractions had been collected. These fractions were adjusted to pH 5 and they were used in treating the growing point of beans.

Good curvatures and malformations were produced on beans treated with the fractions collected from the cation exchanger IR-120 (H). The active substance is apparently not a cation. The results obtained when bean plants were treated with fractions collected from the anion exchanger IRA-400 (OH) were inconclusive. The majority of the plants treated with these fractions showed no effects of the treatment. However, a few scattered plants responded in the usual manner, indicating that the active substance may have passed through the column. When the anion exchange resin was converted to the chloride form and used as described above, the active substance was present in the fractions collected, indicating that the substance is not an anion.

Preliminary Spot Tests: The ether extract (reduced to dryness and taken up in water) of the active filtrate was used in spotting a number of pieces of Whatman No. 3 filter paper. A few preliminary spray experiments have been utilized to learn more of the nature of the compound. Unless otherwise indicated the spray reagents were prepared according to the directions given by Block, Durrum, and Zweig (2). A compound known to give a color reaction with the spray being used was used as a control and in all cases reacted in the expected manner.

1) $FeCl_3$ (2 % aqueous)—No color reaction.
2) $FeCl_3$—perchloric acid (16).
   No color reaction. IAA gave positive reaction.
3) *p*-dimethylaminobenzaldehyde (16).
   No color reaction. IAA gave positive reaction.
4) Ninhydrin—No color reaction.
5) NaOH (1.0 *N*)—No color reaction.
6) Phloroglucinol (3)—No color reaction.
7) Diazotized sulfanilic acid—A very faint orange spot was produced when this spray for imidazole and phenol compounds was used in treating papers spotted with the ether extract of *A. niger* 56-39. Histidine produced a bright red-orange color. Melville (13) has shown that hot water extracts of the mycelium of *A. niger* contain ergothioneine. This compound produces a red color when treated with the diazotized sulfanilic acid. However, this compound is insoluble in benzene, chloroform and ether, solvents which readily extracted the compound described here.

Tests with Other Compounds: Thom and Raper (19) make the following statement concerning *A. niger:* "Molds under this name have been used in literally hundreds of biochemical investigations." It is not surprising, therefore, to find that a larger number of compounds have been reported as being produced by this fungus (see also, 1, 8, 9, 10, 11, 14, 21). A few compounds reported to be produced by *A. niger* or other members of the *A. niger* group have been tested on bean plants and compared with the activity reported here. These were tested at 1000, 100, 10 and 1 ppm and are as follows: urea, oxalic, fumaric, gluconic, pyruvic, ascorbic, gallic, kojic, aconitic, malic, and citric acids, procaine penicillin, and IAA. Also tested were cis-cinnamic, 2,3,5-triiodobenzoic, indolebutyric, 2,4,6-trichlorophenoxyacetic and naphthaleneacetic acids. IAA was tested in water and in the uninoculated corn steep medium with the thought that its activity might be different in the culture medium.

None of the compounds tested were able to duplicate the results obtained when the growing point was treated with the culture filtrate of *A. niger* 56-39. The solubility properties of many of these compounds would also tend to eliminate them as the causative agent.

It must be acknowledged that the biological activity of these pure compounds may be altered when present in a relatively crude culture filtrate extract.

Coleoptile Cylinder Test: Ether extracts (pH 5) of the active culture filtrate of *A. niger* and the uninoculated culture medium were reduced to dryness, brought back to their original volume with 2 % sucrose plus buffer at pH 5.0 (K_2HPO_4 1.794 g/l + citric acid monohydrate 1.019 g/l), diluted to various concentrations in the sucrose-buffer solution, and used in the sensitized coleoptile cylinder test as described by Nitsch and Nitsch (15). Five-mm sections were taken from oat plants, var. Victory grown in vermiculite. Before placing the sections in the test solutions they were floated for three hours on glass-distilled water. IAA was also made up in the sucrose-buffer solutions. The results are presented in table I. No growth stimulation was obtained when sections were treated with various concentrations of an ether extract of *A. niger* culture filtrate, but sections placed in similar extracts of the uninoculated medium showed good increases in length. The lack of growth stimulation by the *A. niger* extract does not mean that auxin is not present. In such an extract other compounds may be present which interfere with the test. Indeed, preliminary experiments indicate that ether extracts of *A. niger* filtrate seriously inhibit IAA induced elongation. Bean plants treated on the growing point with *A. niger* ether extract brought up in the buffer-sucrose solution responded in the usual manner.

Experiments with Other Fungi: During the course of a routine screening program approximately 1500 culture filtrates have been tested for their effects on plants. In no case has the type of activity reported here been observed. Unfortunately, all of these treatments were made on bean plants by treating one of the primary leaves, not the growing point.

TABLE I

MEAN LENGTH OF OAT COLEOPTILE SECTIONS IN SOLUTIONS OF INDOLEACETIC ACID AND ETHER EXTRACTS OF A. NIGER CULTURE FILTRATE AND OF UNINOCULATED CULTURE MEDIUM (CORN STEEP-CERELOSE)

TREATMENT	CONCENTRATION	LENGTH OF SECTIONS (MM)
Buffer-sucrose	....	6.6
IAA	2×10^{-7} M	9.8 **
IAA	2×10^{-8} M	9.9 **
IAA	2×10^{-9} M	8.9 **
IAA	2×10^{-10} M	7.6 **
A. niger (ether extract)	1.0 x *	6.5
"	0.1 x	6.8
"	0.01 x	6.5
"	0.001 x	6.5
"	0.0001 x	6.5
Uninoc. medium (ether extract)	1.0 x *	8.8 **
"	0.1 x	7.7 **
"	0.01 x	6.8
"	0.001 x	6.6
"	0.0001 x	6.7

* Equivalent to the concentration before extraction.
** Significantly different from buffer-sucrose at 1% level.

Approximately 400 of the previously tested and unidentified fungi have been re-examined, therefore, using the methods described herein. In no case has this type of activity been found. In addition, a number of other aspergilli have been re-examined (grown for one and two weeks in shake-flask culture). These were as follows: *A. alliaceus, A. amstelodami, A. avenaceus, A. candidus, A. carbonarius, A. chevalieri, A. clavatus, A. conicus, A. fischeri, A. flavipes, A. flavus, A. fonsecaeus, A. fumigatus, A. giganteus, A. granulosus, A. janus, A. luchuensis, A. melleus, A. miyakoensis, A. nidulans, A. niger, A. ochraceus, A. ornatus, A. oryzae, A. penicilloides, A. quercinus, A. repens, A. ruber, A. rugulosus, A. sydowi, A. sclerotiorum, A. tamarii, A. terreus, A. unguis, A. ustus, A. variecolor, A. versicolor* and *A. wentii*.

With one exception all of the identified species of Aspergillus failed to induce on bean plants the type of response caused by *A. niger* 56-39. A culture of *A. niger* obtained from Professor K. B. Raper (designated as No. 1893) produced the same curvatures and malformations as those produced by the culture designated as 56-39. This would tend to confirm the identification of 56-39 as *A. niger*.

DISCUSSION

When the growing point of a bean plant is treated with the culture filtrate of a fungus identified as *A. niger* marked curvatures and malformations are produced on the developing shoot. In a few cases the stem has been seen to form a complete circle before returning to the normal vertical growth. Curvatures and overgrowths are characteristic of a number of known growth substances, when applied at high concentrations. However, the results obtained here are distinctly different from those produced by known growth substances. The curvatures induced by *A. niger* develop only after the growing point has begun to elongate (3 to 5 days) whereas curvatures induced by IAA are present within 12 hours after treatment. Furthermore, when bean plants are treated with high concentrations of IAA overgrowths occur usually at the site of application. The malformations produced by *A. niger* frequently do not occur until the treated growing point has undergone considerable elongation. Thus, in many cases, the stem may appear more or less normal up to the junction of the 1st compound leaf, and it is here where irregular and abnormal thickenings have been most frequently encountered. Also, when the hypocotyl or epicotyl of bean plants were treated on one side with *A. niger* filtrate no negative curvatures resulted as occurred with IAA. On the contrary, slight positive curvatures seem to be present on plants treated unilaterally with culture filtrates of *A. niger*.

It has been pointed out that early indirect evidence suggests that IAA is produced by *A. niger*. In addition to the evidence cited above the following lines of evidence indicate that the active compound described in this paper is not related to IAA:

1) The effects of the treatment with *A. niger* culture filtrate could not be duplicated by IAA (either made up in water or in the uninoculated corn steep medium).
2) Spray reagents for IAA (and other indole compounds) did not react with active *A. niger* extracts but did with IAA.
3) The active compound produced by *A. niger* is stable to heat in 0.5 *N* HCl and is apparently destroyed by heat in 0.5 *N* NaOH.
4) The active principle is soluble in ethyl ether at pH 2, 5 or 9.
5) The active compound was not absorbed by an anion exchange resin.

Since the ether extract of *A. niger* was only spotted on paper and not chromatographed, contaminating substances might be present which interfere with the color tests. This possibility was explored briefly only with the sprays for indole compounds. It was found that various quantities of indoleacetic acid added to ether extracts of *A. niger* culture filtrate were detectable in concentrations as low as were detectable in similar preparations of IAA made up in water.

The writer has recently described a translocatable plant growth inhibitor produced by *Penicillium thomii* and *Arachniotus trisporus* (7). The inhibitor produced by these fungi differs considerably from the active compound produced by *A. niger*. In the former case, good effects are produced when only one of the primary leaves of a bean plant are treated whereas the *A. niger* compound does not appear to influence the plant when this method of treatment is used. The solubility properties of the *P. thomii* and *A. trisporus* inhibitor differ also from those of the *A. niger* compound. In the former case the inhibitor is insoluble in the fat solvents, while in the latter case the active

compound is easily extracted with fat solvents. No curvatures have been noted following treatment (on the leaf or growing point) of bean plants with the culture filtrate of *P. thomii* or *A. trisporus*.

Experiments are underway to attempt isolation of the active compound as well as to determine further its chemical and physiological properties.

Summary

1. The culture filtrate of a fungus designated as 56-39 and identified as *A. niger* has been found to cause curvatures and grotesque malformations on bean plants 5 to 10 days after the young growing point has been treated. Elongation of the 1st and 2nd internodes above the primary leaves was also strongly inhibited. Malformations have consisted of greatly thickened stems and petioles, irregular stem protrusions, severely twisted stems and stems that are wide and relatively flat. Little or no effect occurs on corn plants at concentrations active on bean plants.

2. When grown on a corn steep-Cerelose medium in shake-flask the active compound begins to appear at the end of two days. It was also produced when the fungus was grown on liquid media of Czapek-Dox, Richard's or potato-dextrose broth but could not be detected in culture filtrates of the fungus grown on the medium of Raulin-Thom.

3. The active principle is stable to heat in 0.5 *N* HCl but is destroyed when heated in 0.5 *N* NaOH. It could be adsorbed onto activated charcoal and eluted with ethanol, acetone or ethyl acetate. The compound is dialyzable. It could not be adsorbed onto cation or anion exchange resins.

4. The active compound is soluble in methanol, ethanol, and *n*-amyl alcohol, acetone, chloroform, benzene, ethyl acetate, and ethyl ether (pH 2, 5 and 9). It could not be extracted with xylene or petroleum ether (B.R. 60 to 70° C).

5. Evidence is presented which eliminates IAA as the active compound. A number of other compounds also known to be produced by *A. niger* have been tested on bean plants. None of those tested have given the response which follows treatment with the culture filtrate of *A. niger* 56-39.

6. Ether extracts of *A. niger* culture filtrate that produced marked curvatures and malformations on bean plants were inactive in a straight growth test using oat coleoptile sections.

The writer is indebted to Prof. K. B. Raper for furnishing many of the identified fungi used in these studies. Prof. J. Tuite assisted in the identification of the culture of *A. niger* for which thanks are given. Able technical assistance was furnished by Mrs. Barbara Shaw.

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INTRACELLULAR LOCALIZATION OF THE TRYPTOPHAN-INDOLEACETATE ENZYME SYSTEM [1,2]

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Tryptophan is degraded by plant tissues or tissue extracts to the auxin indoleacetic acid (IAA) (1). The enzyme system involved is unusually sensitive in vivo to ionizing radiation (2, 4). Since inhibition of the enzyme is detectable in preparations made within several minutes after x-irradiation, it may be inferred that cellular or nuclear replication is not required for expression of this radiation effect. However, the location of the tryptophan-IAA system within the cell and, by implication, the probable cytological locus of the radiation action, were not known. Wildman, Ferri and Bonner (12) had early demonstrated that chloroplast-free preparations of the spinach leaf could convert tryptophan to auxin. Fractionation of tissue breis by differential centrifugation was therefore undertaken to determine specifically the intracellular distribution of the IAA-forming enzyme. It will be shown that this enzyme occurs in preparations of young leaf and meristem cells as a cytoplasmic, submicrosomal system.

METHODS

PLANT MATERIAL: Seedlings of the mung bean plant (*Phaseolus aureus* Roxb.) were used throughout this study. Seeds were sterilized by immersion in 0.3 % Clorpactin solution for five minutes, rinsed, and planted in the gravel of nutriculture beds. The beds were supplied with the nutrient solution described by Nason et al (8) at half strength. Plants were grown in controlled environment rooms under 2500 ft-c of mixed fluorescent and tungsten filament lamps in a wattage ratio of 4 : 1. "Day" and "night" temperatures were, respectively, 27° C. and 19° C. The day length was 18 hours. Only the first primary leaves and bud were taken for fractionation.

FRACTIONATION: For separation of intracellular organelles, tissues were ground in a chilled mortar for several minutes with sand and an equal volume of cold KH_2PO_4-$NaHPO_4$ buffer, 0.2 M, pH 7.0, containing 0.3 M sucrose. The brei was filtered through 200-mesh nylon muslin, and gross debris was removed by centrifugation at $100 \times G$ for 10 minutes. An aliquot of the supernatant ("whole" dispersion) was fractionated by differential centrifugation as indicated in table I. Relative centrifugal forces of $17{,}000 \times G$ and below were obtained with a refrigerated Model SB-1 International centrifuge with high speed attachment. A Spinco Model L preparative centrifuge was used for higher centrifugal forces. Each precipitate was washed once with the sucrose-buffer and recentrifuged. The wash was added to the succeeding fraction, and the pellet was redispersed in sucrose-buffer to the original aliquot volume. In one series of experiments, the sucrose-phosphate was replaced either by 10 % Carbowax 4000 (7) or by water as a dispersion medium. The Carbowax was first purified by precipitation from water by acetone.

For separation of the soluble cytoplasmic proteins by centrifugation, the plant tissues were frozen in liquid nitrogen, pulverized in a mortar, and dispersed in twice their weight of water. Preliminary examination of the soluble fraction in the analytical ultracentrifuge indicated the presence of two major fractions: a fast-moving component with an S'_{20} of about 18 Svedberg units, and a slower-moving heterogeneous component containing substances with sedimentation constants ranging from zero to about four. Therefore, dispersions were first centrifuged at $105{,}000 \times G$ for 30 minutes to remove particulate components. Twelve ml of the resultant supernatant was transferred to each of a pair of centrifuge tubes. These were again centrifuged for 200 minutes at the same relative centrifugal force to throw down a relatively clean preparation of the fast-moving component. In one tube "A" the pellet and supernatant were redispersed ("reconstituted"). The supernatant "B" and pellet "C" in the other tube were separated for analysis. Two runs were made with 7-day and 15-day mung bean seedlings. With the older seedlings, the pellet was dispersed in .01 M NaCl and resedimented into two fractions by centrifugation at $101{,}000 \times G$ for 10 minutes and 240 minutes. All fractions were made to equal volumes with 0.01 M NaCl. This concentration of NaCl was used to give the same equivalent ionic strength as the initial 12-ml volumes of supernatant; effective ionic strength was determined by a conductivity bridge (Industrial Instruments Company, Model RC 16 Bl). Sedimentation patterns were determined with the Spinco Model E analytical ultracentrifuge. Viscosity corrections for water but not the medium were made in calculating sedimentation constants; the values given are all, therefore, S'_{20} constants (10).

ENZYME ACTIVITY: Three-ml aliquots of the various preparations were added to 25-ml Erlenmeyer flasks containing 3 ml 0.85 % (or 4 ml 0.75 %) L-tryptophan in 0.2 M phosphate buffer, pH 7.65; the final pH was 7.40. The mixtures were placed in a Dubnoff shaker at 30° C and covered to exclude light. At half hour intervals each flask was rapidly heated to boiling over a Bunsen flame and kept at that temperature for one minute. Neutral and basic lipophilic substances were extracted from the various heat-denatured incubation mixtures with ether. Each aqueous phase was then acidified to pH 3.0 (glass elec-

[1] Received July 16, 1957.

[2] This work was performed under the auspices of the U. S. Atomic Energy Commission.

trode) and the acid fraction was extracted, also with ether.

Initially the acid fractions were assayed by direct colorimetry. Although the results were essentially in agreement with those obtained by assay after chromatographic resolution of the IAA, we feel that direct colorimetry is in many instances qualitatively and quantitatively unreliable as a measure of either auxin or IAA concentration in impure extracts (5). Accordingly, for the data reported here, acid fractions were first resolved by paper chromatography (5). The IAA spot was eluted with methanol and assayed colorimetrically with the Salper reagent (5) or, where indicated, biologically with the Avena curvature test (6). Sand-grown plants were used for the bioassay; curvatures in the proportional range were converted to IAA equivalents using response curves with pure IAA that were determined at the same time.

Enzyme activities are expressed as the slopes of the empirical activity curves for each fraction (milli-micrograms (mμg) IAA found per ml enzyme vs. time in minutes within the incubation period of two hours). It is felt that these values represent enzyme activities at substrate saturation levels; the curves are linear up to 120 minutes, and no reduction in yield was obtained when a whole dispersion was incubated with tryptophan at 0.6 the concentration level normally used.

Nitrogen Determinations: Aliquots, usually 0.5 ml, of the various preparations were added to an equal quantity of chilled 10 % trichloroacetic acid (TCA). The resultant precipitate was washed twice by centrifugation with 5 % TCA. It was then digested with H_2SO_4 and cleared with 30 % H_2O_2. After partial neutralization of the acid, ammonia was determined by direct nesslerization.

Results and Discussion

The distribution of enzyme activity in sucrose-phosphate dispersions of the leaf and bud tissues is given in table I. Both colorimetric and biological determinations of the IAA produced indicate that the major fraction of the tryptophan-IAA activity occurs as a "soluble" component of the cytoplasm. Relatively little, if any, of the activity is associated with the macro-particulates brought down by centrifugation. Comparison of the activities of the reconstituted fraction and the supernatant indicates that no component inhibitory to the enzyme is associated with a gross particulate. Conversely, neither is there any particulate activity that is masked or inhibited by the presence of other cellular fractions. Table I also shows that the activity of the soluble fraction on a nitrogen basis is roughly twice that of the whole dispersion. This would be expected since most of the

Table I

Conversion of Tryptophan to Indoleacetic Acid by Cell Fractions of the 7-day-old Mung Bean Seedling

Fraction	Centrifugation	Description	Nitrogen mg/ml	Activity *				Specific activity **	
				Colorimetry	%	Bioassay	%	Colorimetry	Bioassay
	10′ 100 × G ↓								
A	Supernatant	"Whole dispersion"	1.13	9 13	100	15	100	10	13
A_1	"	Boiled 5′	1.12	0	0	0.33	2	0	0.3
A_2	"	Without tryptophan	1.13	..	..	< 0.01	< 0.1	..	< 0.01
B	10′ 1,000 × G	Plastids, nuclei wall fragments	0.054	0	0	0.27	2	0	5
C	10′ 4,500 × G	Plastids, nuclei	0.120	0	0	0.05	0.3	0	0.4
D	30′ 17,000 × G	Mitochondria, plastids	0.181	0	0	0.44	3	0	2
E	30′ 105,000 × G ↓	"Microsomes"	0.115	0	0	0.17	1	0	1
F	Supernatant	Soluble protein	0.602	8 9	75	18	120	14	30
G	.....	Reconstituted (B, C, D, E, F)	1.07	9 9	80	12	80	8	11

* mμg IAA/min × ml enzyme.
** mμg IAA/min × mg nitrogen.

TABLE II

CONVERSION OF TRYPTOPHAN TO INDOLEACETIC ACID BY DISPERSIONS OF 10-DAY-OLD SEEDLINGS IN CARBOWAX, WATER, AND SUCROSE-PHOSPHATE

FRACTION	IAA (MμG/MIN × ML ENZYME)		
	CARBOWAX	SUCROSE-PHOSPHATE	WATER
Whole dispersion	8	14	17
↓ 105,000 × G 30′			
Pellet	Trace	0	0
Supernatant	9	11	20

enzyme activity occurs in the fraction that contains 50 % of the TCA precipitable nitrogen of the cell.

The tryptophan-IAA system may be linked to cytoplasmic inclusions by unstable bonds, or be associated with particularly fragile particles. If so, we cannot exclude the possibility by the present approach that the preparative method liberates the enzyme into the supernatant fluid (Hele in (3)). However, it is pertinent that the use of polyglycols as an isoosmotic dispersion medium, or dispersion in water, yielded results qualitatively identical to those obtained with sucrose-phosphate buffer. Table II indicates that the full activity of the whole dispersion is found in the supernatant fraction following centrifugation of dispersions in these media, even though the total activity was lower with the glycol and higher with the water as compared to the sucrose-phosphate.

The enzyme activities listed in tables I and II represent the amounts of IAA formed from tryptophan by preparations heterogeneous in molecular composition. They represent net or integral values. On the other hand, the low rates of tryptophan conversion found for the macro-particulate fractions conceivably might result from the action of concomitantly functioning systems for IAA inactivation. The similar yields from the reconstituted dispersions, supernatant fractions, and whole dispersions (tables I and II) tend to disprove this interpretation.

To evaluate further the possibility of concomitant IAA destruction, a known amount of IAA was added to the various centrifugal preparations described in

TABLE III

RECOVERY OF INDOLEACTEIC ACID ADDED TO THE VARIOUS INTRACELLULAR FRACTIONS DESCRIBED IN TABLE I

FRACTION	IAA RECOVERED (μG)	%
Control (33 μg IAA in sucrose-phosphate)	27	100
A	25	92
A_1	29	104
B	24	89
C	25	92
D	29	104
E	30	106
F	22	81

TABLE IV

TRYPTOPHAN CONVERSION TO INDOLEACETIC ACID BY CYTOPLASMIC PROTEIN FRACTIONS OF THE MUNG BEAN SEEDLING

FRACTION	PROTEIN * MG/ML	ACTIVITY **	SPECIFIC ACTIVITY ***	APPARENT PROTEIN CONC † MG/ML ENZYME			S'_{20} OF FAST COMPONENT
				"SLOW"	"FAST"	TOTAL	
7-day plants							
IA-Reconstituted	6.4	16	16	2.4	1.7	4.1	17.5 ††
IB-Supernatant	2.8	14	31	3.1	0.4	3.5	17.7 ††
IC-Pellet	2.8	0	..	0	1.2	1.2	18.1 ††
15-day plants							
IIA-Reconstituted	2.9	22	48	3.2	1.0	4.2	18.6 ‡
IIB-Supernatant	1.6	25	96	3.5	0.3	3.8	19.2 ‡
IIC-Pellet	1.2	< 2	< 10	0	1.0	1.0	18.8 ‡
↓ Recycle		2.5	12				
IIC-1 Pellet (10′, 101,000 × G)	.11	0					
IIC-2 Pellet (240′, 101,000 × G)	.12	0					
IIC-3 Supernatant (240′, 101,000 × G)	.50	0					

* TCA precipitated N × 6.25.
** mμg IAA/min × ml enzyme.
*** mμg IAA/min × mg N.
† Calculated from planimetric measurements of peak areas on sedimentation plates, assuming a refraction increment of bovine serum albumin (10).
†† Temperature during sedimentation averaged 27.7° C.
‡ Temperature during sedimentation averaged 20.9° C.

table I. After two-hour incubation and partition treatments identical to those used for the determination of tryptophan conversion activity, the residual concentrations of IAA in the acid fractions were estimated by colorimetry after chromatographic resolution of the extract. The results are given in table III. They indicate that from 90 to 105 % recovery was obtained with the particulate fractions, whereas the recovery of IAA from the supernatant fraction was about 80 %. Thus table I may be considered to give a conservative estimate of the relative distribution of tryptophan conversion activity in the various intracellular fractions.

Further localization of the enzyme system within the proteins of the cytoplasm was achieved through ultracentrifugation of seedling dispersions as detailed under METHODS. Sedimentation patterns of the various preparations are shown in figure 1, and the activity and characteristics of the fractions are given in table IV. Figure 1 demonstrates that the cytoplasmic proteins of the mung bean seedling, like those of other higher plants (13) or the animal (9), fall almost wholly into two major fractions that are distinguishable in the ultracentrifuge. One fraction, the pellets IC and IIC in figure 1, behaves as an almost homogeneous entity on sedimentation, with an S'_{20} of about 19. It comprises about 60 % of the cytoplasmic protein. Table IV shows that essentially no IAA-forming activity is associated with this fast-moving component. If the slight apparent activity in the pellet of the 15-day plants is derived from enzyme adsorbed to the heavy protein, the second sedimentation run shows that enzyme elution was not accomplished by redispersion in neutral 0.01 M NaCl.

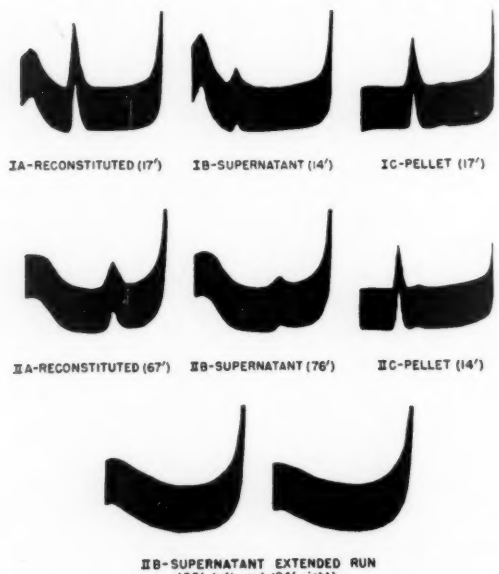

FIG. 1. Sedimentation patterns of cytoplasmic protein fractions of 7-day (I) and 15-day (II) mung bean plants. The numbers in parentheses represent the minutes at 59,780 rpm.

Some of the fast-moving component remains in the supernatant after centrifugation as a residual contaminant (IB and IIB, fig 1). From the estimates of protein concentration based on area measurements of the sedimentation plates (table IV), it is about one-fourth of the supernatant protein from both the 7- and 15-day plants. The remaining moiety of the supernatant is composed of substances of S′ between zero and about four. Its heterogeneity is indicated by the extended run sedimentation patterns (92 and 124 minutes) of the IIB supernatant in figure 1. This lack of resolution would be consistent with particles in a graded progression of sedimentation velocities. According to the conversion rates given in table IV, it is this heterogeneous, slow-moving fraction that contains essentially all of the enzyme that converts tryptophan to IAA. The similar IAA yields from the reconstituted and supernatant preparations make it improbable that the two fractions form a coupled system for tryptophan conversion.

A small amount of a component that migrates faster than the main peak may be observed in IC and IIC of figure 1. It has an S′ of 28.4. Possibly it is a dimer of the S′19 protein (cf (13)); a dimer with an S′ in the region of 27 might be anticipated from S′19 globular molecules. All we may conclude here, however, is that no appreciable tryptophan-conversion activity is associated with this minor fraction that sediments most rapidly, since it would be found in the IC and IIC pellets that table IV indicates as having little or no activity.

It is highly probable that the S′19 component here examined is identical with the cytoplasmic "Fraction I" protein characterized by Wildman and co-workers (13). Wildman and Bonner (11) stated that Fraction I protein did not possess tryptophan-to-auxin activity. On the basis of the present work we may conclude that the Fraction I protein has no appreciable tryptophan-to-IAA activity, and that essentially all of this enzyme activity in leaf and bud tissues is associated with the heterogeneous cytoplasmic complex identifiable ultracentrifugally as the S′0-4 fraction.

A case can be made to support the hypothesis that the native auxin of hormonal function in the higher plant is IAA, and that IAA is normally synthesized from tryptophan (1, 2). If we accept this concept, it then follows from the present study that the locus of auxin biogenesis is cytologically continuous and contiguous to the functional, and possibly primary, site of auxin action in growth: the cytoplasmic membrane and cell wall. The present observations tend also to support the inference that the locus of radiation damage to enzymatic biosynthesis of auxin, and more specifically, of IAA, is in the cytoplasm. The implications of such a localization to the apparent channelling of absorbed high energy radiation to specific biochemical transformations have been discussed elsewhere (3, 4).

Summary

An analysis of the intracellular distribution of the enzyme that converts tryptophan to indoleacetic acid has been made for the mung bean seedling. Cellular fractions were separated by differential centrifugation and their indoleacetate-forming activities were determined by chromatographic resolution of the auxin. It is concluded that the tryptophan-to-indoleacetate enzyme system occurs as a soluble component of the cytoplasm, and that it is in the group of heterogeneous macromolecules with sedimentation coefficients between zero and four Svedberg units.

The participation of Dr. Clifford Sato at the start of this investigation is gratefully acknowledged. I also wish to thank Dr. Arthur L. Koch and Mr. William A. Lamont for their help in determining the sedimentation patterns, and Mrs. Marcia Eib for her technical assistance. A preliminary summary of this work has appeared (3).

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OXIDATIVE PHOSPHORYLATION AND FUNCTIONAL CYTOCHROMES IN SKUNK CABBAGE MITOCHONDRIA[1,2]

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The extremely rapid respiration of the spadix of certain members of the Araceae has attracted special attention because of its insensitivity to such classical inhibitors as cyanide and carbon monoxide. Although it was concluded that a flavoprotein serves as the terminal oxidase in such tissues (15), it is now clear that they contain high concentrations of cytochromes which probably function in respiration (2, 4, 12, 20, 22, 25). Following the demonstration that isolated spadix mitochondria oxidize Krebs cycle acids very rapidly (13), it was shown that most of the cytochrome components are also localized on these particles (2, 4, 12). The mitochondria contain enough cytochrome c oxidase (22) and diphosphopyridine nucleotide (DPNH) oxidase (12) to account for essentially all of the tissue respiration. The conclusion that terminal respiration in the Aroid spadix, like that in other plant tissues (11), is mediated by the particle-bound cytochrome system is supported by spectrophotometric observations of the intact tissue (25).

The above findings do not indicate whether the rapid consumption of oxygen by spadix tissue is associated with the supply of utilizable energy to the cells. Is the respiration normally linked with phosphorylation and synthesis, or is it "uncoupled" and used for some other purpose, such as the generation of heat? To answer this question, it is first necessary to know whether the mitochondria are able to couple substrate oxidations to phosphorylations. Preliminary determinations indicated a very low efficiency of oxidative phosphorylation (13) and the present study was undertaken to obtain further evidence on this point. An attempt has also been made to examine quantitatively some of the cytochromes involved in

[1] Received July 23, 1957.

[2] This work was supported by a grant from the National Science Foundation.

the reaction of the mitochondria with oxygen. The effects of inhibitors were studied in order to explore the mechanism of the cyanide-resistant respiration.

Materials and Methods

Young skunk cabbage (*Symplocarpus foetidus*) spadices were collected either before the stamens had emerged from between the sepals or shortly thereafter and stored in the refrigerator (4° C). Only the flowers, cut away from the central axis, were used in the experiments. Cell fractions were isolated by differential centrifugation of tissue homogenates, prepared by grinding in a glass mortar with sand, and all operations were carried out at roughly 0° C. The grinding medium contained 0.5 M sucrose, 0.05 M TRIS (tris hydroxymethylaminomethane) buffer (pH 7.0), and 0.01 M neutralized EDTA (ethylenediamine tetraacetate) when particles were to be used in the manometric studies; otherwise, it contained 0.5 M sucrose and 0.05 M phosphate buffer (pH 7.0). The homogenate was strained through cheesecloth and centrifuged at 1,000 × G for 5 minutes to remove sand and cell debris. The mitochondrial fraction was isolated by centrifuging at 10,000 × G for 15 or 20 minutes, and washed by resuspending in the isolation medium and recentrifuging. Particles to be used in the spectrophotometric studies were washed twice in this manner. The washed pellet was suspended in a small volume of 0.5 M sucrose (for manometric studies) or 0.5 M sucrose–0.05 M phosphate buffer.

Oxygen uptake was measured at 30° C using conventional Warburg manometric techniques. The substrate, usually α-ketoglutarate, was tipped in after 10 minutes of equilibration. Phosphate esterification was measured by determining the disappearance of inorganic phosphate from the reaction mixture, to which glucose and hexokinase had been added. Fluoride was included to prevent hydrolysis of ATP (adenosine triphosphate) and malonate to block oxidation at the level of succinate. The cofactors added were: DPN, ADP (adenosine diphosphate), DPT (diphosphothiamine), and CoA (coenzyme A). The reaction was stopped after the appropriate interval by tipping in 0.5 ml of 20 % trichloracetic acid. Inorganic phosphorus was determined by the method of Bernhart and Wreath (3).

For the spectrophotometric measurements, an appropriate volume of mitochondrial suspension (equivalent to roughly 5 g of initial tissue) was transferred to a Lazarow and Cooperstein cell (17); buffer was added to give a final concentration of 0.025 M phosphate and 0.25 M sucrose in 3 to 4 ml. Substrate and/or inhibitor were added as the solid sodium salts of citrate, cyanide, or azide. The cell was then gassed for 10 minutes with nitrogen and the stopcocks closed. The absorption spectrum of the turbid suspension was determined in a Beckman DU spectrophotometer, equipped with photomultiplier attachment, using filter paper as the blank. Typical slit-widths were 0.05 mm for 400 to 500 mμ, 0.02 mm for 500 to 600 mμ, and 0.03 mm for 600 to 630 mμ; these values would give representative band widths of 1.5 mμ at 450 mμ, and 1 mμ at 550 mμ. The spectrophotometer was calibrated against mercury emission lines (Beckman mercury lamp). Following a determination of the spectrum either above or below 500 mμ, the cell was gassed for 10 minutes with oxygen, and another spectrum recorded. Difference spectra were obtained by subtracting the absorbance in oxygen from that in nitrogen. During the measurement of the spectra there was a slight decrease in absorbance at all wavelengths, possibly due to swelling or aggregation of particles; since the time of measurement (roughly one half hour) after gassing with N_2 or O_2 was essentially constant, this factor probably does not affect the difference spectra greatly.

Results

Oxidative Phosphorylation: Washed skunk cabbage mitochondria show essentially no endogenous respiratory activity, but they take up oxygen rapidly in the presence of Krebs cycle acids; with α-ketoglutarate, like succinate (12), the rate tends to fall off after the first 15 minutes. Table I shows the results of four experiments in which the oxygen uptake and phosphate esterification were determined in the presence of α-ketoglutarate. Although there is some variation in the results, phosphate/oxygen (P/O) ratios of greater than three were obtained in each experiment. In two other experiments, all the P/O values were between two and three. In general, the lower P/O ratios were correlated with higher oxygen uptake, suggesting that these preparations may have

Table I

Oxidative Phosphorylation with α-ketoglutarate

Experiment	Oxygen uptake μ atoms/hr × vessel	Phosphate esterified μ atoms/hr × vessel	P/O
1	3.84	13.55	3.53
	4.55	15.29	3.37
	2.23	6.88	3.08
2	3.98	15.00	3.78
	3.58	13.25	3.70
	5.14	13.65	2.65
	5.75	14.81	2.59
3	1.43	5.03	3.52
	2.14	7.16	3.35
4	2.58	9.65	3.74
	3.12	11.42	3.66
	2.58	9.28	3.60

Contents of complete reaction mixture (in micromoles): substrate—60, phosphate—30, $MgSO_4$—1.8, ADP—3, DPN—1.2, DPT—0.2, CoA—0.1, NaF—30, malonate—6, glucose—60, and hexokinase—1 mg in a total of 3 ml. pH = 7.0. Each vessel contained mitochondria from approximately 3.3 g of tissue. Temp—30° C; gas phase—air; duration of experiment—30 minutes for 1 and 2, 20 minutes for 3 and 4.

TABLE II

EFFECTS OF DNP, AZIDE, AND CYANIDE ON OXIDATIVE PHOSPHORYLATION

EXPERIMENT	NUMBER OF VESSELS	INHIBITOR M CONC	O_2 UPTAKE μ ATOMS *	P_i ESTERIFIED μ ATOMS *	P/O
1	3	0	9.3	21.00	2.19
	2	10^{-5} DNP	7.4	3.78	0.53
	2	10^{-4} DNP	6.5	2.08	0.33
2	3	0	2.76	10.12	3.67
	2	10^{-3} Azide	1.9	4.84	2.54
	2	10^{-2} Azide	1.0	2.52	2.52
3	3	0	5.63	14.51	2.58
	2	10^{-3} Azide	3.91	7.36	1.89
	1	10^{-2} Azide	3.31	5.61	1.69
4	2	0	1.73	6.10	3.44
	3	4.6×10^{-4} Cyanide **	2.15	3.39	1.58

For contents of reaction mixture, see table I.
* Average value per vessel.
** KCN in main compartment and appropriate KCN-KOH mixture in center well.

been partially uncoupled. Omission of malonate from the medium in one experiment did not significantly alter the ratio. The average value for the P/O in all the experiments was 3.17, with a maximum of 3.78.

In order to verify the fact that the measured changes are due to oxidative phosphorylation, the effect of the uncoupling agent 2,4-dinitrophenol (DNP) was tested. Table II shows that low concentrations of DNP cause a drastic inhibition of phosphorylation, even though oxygen uptake is only slightly reduced. The small amount of phosphorylation remaining may be linked to substrate oxidation, since this step is not sensitive to DNP. This inhibitor is known to uncouple only the three phosphorylations associated with the oxidation of DPNH by O_2 (14).

The effects of two agents, azide and cyanide, which are used to block electron transport in the cytochrome system were determined. It has been shown that organic acid oxidations by spadix mitochondria are relatively insensitive to cyanide (12, 16, 22). Similarly, citrate and α-ketoglutarate oxidation by skunk cabbage particles are only inhibited approximately 30 % by 10^{-3} M azide. Table II shows that azide, at 10^{-3} and 10^{-2} M, inhibits phosphate esterification considerably more than O_2 uptake, so that the P/O value is lowered by roughly one unit. This was true whether the control value was less than or greater than three. A concentration of cyanide which had no effect on the oxygen uptake markedly inhibited the phosphorylation (table II); the decrease in P/O ratio suggests that two phosphorylative steps have been uncoupled. Although the cyanide results represent a single experiment, the replication was very good.

SPECTROPHOTOMETRIC OBSERVATIONS: The absolute absorption spectrum of a mitochondrial suspension shows a high absorbance between 400 and 500 mμ and relatively little at longer wavelengths. There are three major absorption bands, at 420 to 430, 440 to 450, and 475 to 480 mμ; the position and height of the first two bands are altered on going from the reduced to the oxidized state. A slight elevation in the absorbance at 550 to 560 mμ can be detected when the particles are in nitrogen. These spectra are very similar to those obtained with deoxycholate-solubilized skunk cabbage mitochondria (12).

To reveal the nature of the components which are involved in the reaction with molecular oxygen, difference spectra (N_2-O_2) were calculated and a typical curve from 400 to 500 mμ is shown in figure 1. In this region there are two major peaks, at 426 to 428 and 443 to 445 mμ; in both cases, the slight asymmetry suggests that more than one component may be

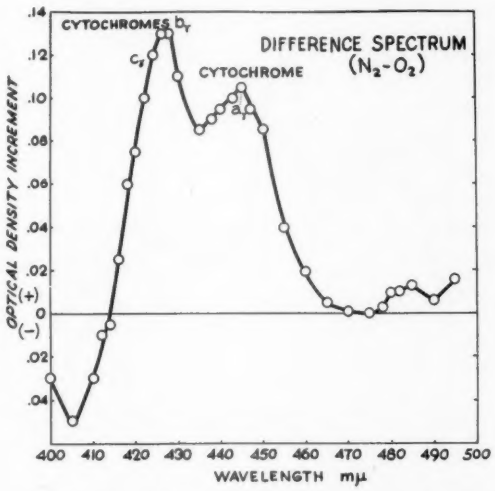

FIG. 1. The difference spectrum of a mitochondrial suspension gassed initially with N_2 and then with O_2.

represented. The first peak probably represents the combined bands of cytochromes of the b and c type, whereas the second corresponds well with the cytochrome oxidase (a_3) maximum. A smaller band at 480 mμ, with two apparent peaks, is also present in the difference spectrum. Although its nature has not been established, the fact that carotenoids absorb strongly in this region suggests that they may be altered when O_2 is added.

Figure 2 shows a difference spectrum from 500 to 630 mμ plotted on a wider optical density scale. The band centered around a peak at 525 mμ, with shoulders on each side, can be attributed to the combined β-bands of cytochromes c and b. There is a broad band from 543 to 565 mμ, with a maximum at 553; this band shows marked asymmetry towards the longer wavelengths, including humps at 558 and 562 mμ. These peaks represent the α-bands of cytochromes of the c and b type, with at least two b's suggested. The third band, with a peak at 602 to 605 mμ, corresponds well with the a maximum of cytochrome oxidase (a-a_3). Using values for the absorption maxima and isobestic points (reference wavelengths) determined for similar animal cytochromes (9), the amounts of the various cytochromes involved in the reaction with oxygen were computed (table III). The concentrations of functional cytochromes are of the order of 10^{-6} M; since the volume of suspension employed corresponded roughly to the volume of tissue from which the particles were obtained, similar intracellular concentrations are indicated. Bendall and Hill (2) have described a cytochrome b_7, with absorption maximum at 560 mμ, in mitochondria isolated from the sterile portion of *Arum maculatum* spadices. If this value of 560 mμ is substituted for the 564 mμ maximum of b, the calculated cytochrome concentration is 1.4 micromoles; this suggests that there may be considerably more b_7 than b in the particles. If the wavelength maximum at 553 mμ is used for the calculation of the amount of c-type cytochrome, a value of 1.42 micromoles is obtained. This may give an indication of the content of cytochrome c_1, which has an absorption maximum at 554 mμ (24), assuming it shows the same molecular extinction change.

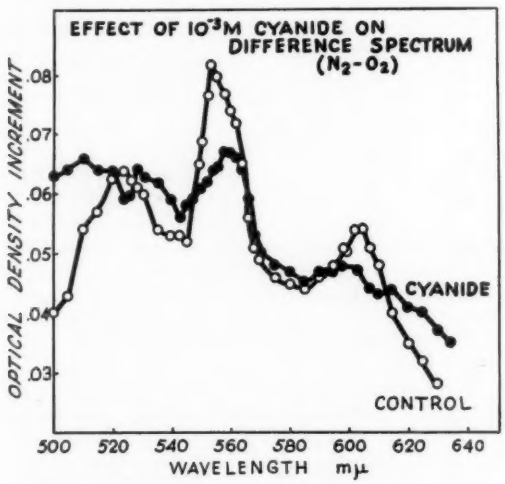

FIG. 2. The effect of cyanide on the difference spectrum of a mitochondrial suspension. The absolute spectra for the control in N_2 and O_2 were recorded, after which NaCN was added and the recordings repeated.

TABLE III

FUNCTIONAL AMOUNTS OF CYTOCHROMES DETERMINED FROM DIFFERENCE SPECTRUM

Cytochrome	a	b	c
Maximum λ	605	564	550
Reference λ	630	575	540
O.D. (cm^{-1})	.026	.019	.016
Conc (micromoles) *	1.62	.95	.84

* Computed from $\Delta\Sigma$($cm^{-1} \times$ millimoles^{-1}) values of 16, 20, and 19 for cytochromes a, b, and c respectively (9).

The effect of 10^{-3} M cyanide on the difference spectrum (N_2-O_2) is also shown in figure 2. The change in the a-band of cytochrome oxidase indicates that cyanide has largely blocked its reaction with O_2; the inhibition averaged about 80 %. The shape of the 550- to 565-mμ band is markedly altered, the major peak now being at 560 to 562, rather than at 553 mμ. This means that although oxidation of the c-type cytochrome is largely inhibited (80 %), some cytochrome b is relatively unaffected (oxidation only about 20 % inhibited). Similar results were obtained with three separate preparations. This suggests that when the cytochrome c–oxidase system is largely inhibited, most of the cytochrome b can still be oxidized by O_2. The 3rd absorption band in this region also shows a slight shift towards a longer wavelength, suggesting a relatively greater participation of b, with its peak around 530 mμ. The cause of the change in the difference spectrum around 500 mμ is not known, although flavoproteins do absorb in this region. In the Soret region, cyanide caused a marked reduction of the 445-mμ peak (a_3) and changes in the 410- to 430-mμ peak which suggest less inhibition of cytochrome b than c; this evidence was limited to a single difference spectrum.

Preliminary experiments were carried out with other inhibitors. In two cases, 10^{-2} M azide reduced the functional amount of cytochrome oxidase by 55 and 72 %, as calculated from the absorbance at 445 minus 455 mμ. Carbon monoxide was bubbled through an anaerobic suspension of mitochondria and the absorption determined. The difference spectrum (N_2 + CO) – (N_2) showed a peak around 428 mμ, which is close to the correct position for the cytochrome a_3-CO complex (6).

DISCUSSION

The maximum P/O values that have been obtained for α-ketoglutarate oxidation by animal mitochondria approach four (14). In recent work, P/O ratios greater than three have been reported for mitochon-

dria isolated from etiolated peas (23), sweet potatoes (18), and castor bean endosperm (1), although the values for plant particles are generally lower (11). The unusually high values reported here (average 3.17, maximum 3.78) suggest that the phosphorylation mechanisms in skunk cabbage mitochondria are as efficient as those in other comparable plant and animal particles. The very low values obtained previously with *Arum maculatum* (13) probably resulted from the use of inadequate isolation methods. The demonstration that isolated particles possess a high capacity for phosphorylation does not prove that they operate with maximum efficiency in vivo, although this is a reasonable hypothesis. Assuming that the mitochondria are the normal respiratory centers, the results suggest that the very rapid respiration of the Aroid spadix can be coupled to energy-requiring processes.

The difference spectra show clearly the participation of cytochromes of the a, b, and c types in the reaction of skunk cabbage mitochondria with oxygen. The positions of the peaks in the α and Soret regions, the reaction with CO, and the previous demonstrations of cytochrome oxidase activity (12, 22, 25) all suggest that a typical cytochrome a-a_3 complex is functioning in these particles. As yet, no other a-type oxidase has been found. The difference spectrum in the 560 mμ region suggests that more than one cytochrome b is involved, in agreement with the spectroscopic observations of Bendall and Hill (2). The fact that the major peak in the α-region is at 553 mμ, rather than at 550 mμ, suggests that the particles may contain both cytochromes c and c_1, with a preponderance of the latter. A recent report indicates that this may also be the case in other plant mitochondria (20). Like the comparable animal particles (9), skunk cabbage mitochondria contain more cytochrome a than b and c (table III). However, they apparently differ by having relatively high concentrations of other components, described here as cytochromes b_7 and c_1; the total concentration of b's is probably greater than a. Although the exact roles of the various components have not been defined, they are almost certainly involved in the oxidative phosphorylations. The fact that the P/O ratio can approach four suggests that the normal electron transport proceeds from the level of cytochrome b to c to a to oxygen, as in animal mitochondria (8).

The oxidation of organic acids by isolated Aroid spadix mitochondria is relatively resistant to cyanide and azide. Although James and Elliott (16) observed little or no inhibition by 10^{-3} M cyanide, subsequent experiments have indicated that this concentration reduces the oxidations between 50 and 60 % (12, 22); in this study, there was no inhibition by 4.6×10^{-4} M HCN (table II). This is in marked contrast to the great sensitivity of the cytochrome oxidase activity, which is essentially completely blocked by 10^{-4} M HCN (12, 22, 24). As suggested by Simon (22), a possible explanation for this discrepancy is that there is an internal cytochrome oxidase, which is inaccessible to added cytochrome c and is not readily inhibited by cyanide; in other words, there is a sufficient excess of the oxidase to account for the cyanide-resistant particle (and tissue) oxidations. Some support for this hypothesis may be gathered from the fact that the difference spectra reveal only an 80 % inhibition of the oxidase by 10^{-3} M HCN; the remaining 20 % might maintain the maximal respiration rate. An alternative explanation is that there is a separate, cyanide-resistant respiratory pathway, which might be functional only in the presence of the inhibitor. This alternate, internal pathway would only be available to added organic acids and not to added cytochrome c. In this connection, the relatively small cyanide inhibition of the functional cytochrome b (fig 2) suggests that it could participate in such a pathway. One of the properties of the mitochondrial cytochrome b_7 is its rapid autoxidation in the presence of cyanide (2). It is not yet known whether this reaction between cytochrome b and O_2, which may involve other components (7), is sufficiently rapid to account for all the cyanide-resistant respiration. Spectrophotometric observations on intact spadix tissues have also shown that cytochrome b can be oxidized in the presence of cyanide, and its possible respiratory role has been considered (25). The striking similarity between the difference spectra obtained with intact tissues and isolated mitochondria supports the assumption that these particles are the normal respiratory centers.

The effects of inhibitors on the oxidative phosphorylation may throw some light on the respiratory pathways. Both azide and cyanide act as "uncoupling agents," reducing phosphorylation more than oxygen uptake (table II). If the relative insensitivity of the respiration to these agents were due solely to an excess of cytochrome oxidase, it is not clear why they should lower the P/O ratio. A possible explanation is that they inhibit the phosphorylation mechanism itself, as well as the terminal oxidase. This is probably the case with azide, which is known to interfere with phosphorylation (19) and the exchange of inorganic phosphate (P^{32}) with ATP (21). No comparable effect on phosphorylation has been reported for cyanide; it causes relatively little inhibition of the P^{32}-ATP exchange (5). An alternative explanation is that the inhibitor diverts the electron transport from the normal pathway to an insensitive, non-phosphorylating pathway, which does not play an appreciable role in the absence of inhibitor. The spectrophotometer evidence suggests that the "switch-over" may take place in the region of cytochrome b. Since there are probably two phosphorylative steps between cytochrome b and O_2 (10), it is suggestive that cyanide apparently uncoupled two of the four phosphorylations associated with α-ketoglutarate oxidation (table II).

Summary

The mitochondrial fraction isolated from skunk cabbage flowers can couple the oxidation of α-keto-

glutarate to the esterification of inorganic phosphate. The high P/O ratios (average 3.17, maximum 3.78) indicate that this oxidative phosphorylation is as efficient as that shown with other plant and animal mitochondria. The phosphorylation can be uncoupled from respiration to a large extent by 2,4-dinitrophenol and to a lesser extent by azide and cyanide. The difference spectrum (N_2-O_2) of a whole mitochondrial suspension shows maxima characteristic of cytochromes a, b, and c, with evidence of relatively high concentrations of b_7 and c_1. In the presence of cyanide, the spectrum shows a large inhibition of c and a, but b is relatively unaffected. The possible role of a b-type cytochrome in the cyanide-resistant respiration is considered.

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TRANSLOCATION OF SUGARS IN THE CONCORD GRAPE[1,2]

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For many years there has been a reasonable consensus among plant physiologists that sucrose is the principal transport sugar in various species of plants (5, 11, 12, 17, 18). A considerable number of investigators, however, have ascribed to hexoses the quantitatively more significant role in translocation (3, 14; see also literature review in Leonard, 10). A possible conclusion from these studies is that the relative translocatability of the various sugars varies significantly with species, and perhaps with environmental conditions as well.

The analysis of this problem is admittedly complicated by the difficulty of distinguishing between the molecules in the phloem actually in transit from the leaves or other supply centers and molecules of the same species originating in metabolic reactions in this tissue, or captured in metabolic pools of the respective sugars in the non-conducting cells of the phloem and closely adjacent tissues. However, the techniques of radiochemistry and chromatography now permit a more definitive analysis of this problem, and a re-examination, therefore, of certain aspects of sugar translocation has been undertaken. The grape was chosen for the initial studies in this series because of the ease with which the bark and wood may be separated for analytical purposes, and because of the extensive information available on the anatomy and cytology of its phloem (6).

MATERIALS AND METHODS

Three-year-old grape stocks (*Vitis labruscana* var. Concord) were planted in April in 12-inch pots, and grown under greenhouse conditions until the plants were used for experimental purposes (July-August). At this time the canes were about 10 feet in length. During the growing period, the plants were trained to a maximum of four canes each, without laterals, either fruiting or vegetative.

Prior to each experiment, the plant selected for experimental purposes was pruned to two canes, transported to a controlled environment room (22° C, 2000 ft-c, 16-hr photoperiod) and left for an adjustment period of 24 to 48 hours. At the end of this period, the youngest fully-expanded leaf on one of the canes (usually a leaf at a distance of 100 to 120 cm from the shoot tip, and having an area of approximately 250 cm^2) was enclosed in a glass chamber modified from a three-liter Florence flask and supplied with $C^{14}O_2$. Details of the experimental set-up are shown diagrammatically in figure 1. Sufficient C-14 labeled $BaCO_3$, as well as carrier carbonate, were weighed out into flask C to provide an initial level of approximately 2 mc of activity in approximately 0.1 % CO_2. A small diaphragm pump (G) was used to circulate the gas through the system, which had a volume of approximately 55 liters.

The system was pressure-tested at about 20 cm of water pressure prior to the start of each experiment to test for possible leaks. The petiole of the C-14 leaf was encased in a split rubber stopper inserted in the leaf chamber and sealed with a commercially available mastic compound ("Kalk-Kord"), a preparation known to be highly impervious to CO_2.

Although photosynthesis proceeded rapidly (in each experiment, 50 % of the labeled carbon dioxide was absorbed from the system in approximately two hours, corresponding to a calculated average rate of CO_2 uptake by the leaf of about 10 mg/dm^2 × hr for this time period) an interval of some 6 to 12 hours elapsed in the different experiments before C-14 activity could be detected at the stem node of the C-14 leaf with a portable 1.5 mg/cm^2 end-window counter. Following this time lag, translocation towards the stem tip was very rapid, activity being detectable at the tip within two hours. Thus a velocity of at least 1 cm/min was indicated for the acropetal translocation rate. Basipetal translocation was much slower.

Shortly after radioactivity could be detected in the shoot tips, the experiment was terminated and segments 30 mm long were rapidly harvested from various internodes, separated into bark and wood, and immediately frozen in liquid nitrogen. All samples were then lyophilized and sealed into glass ampoules under vacuum for storage until analyzed. Although samples were harvested from internodes both basipetal and acropetal to the supply leaf, only the latter will be here reported.

Preparatory to analysis, the tissue samples were pulverized in a micro-mortar, and an accurately weighed sample of approximately 30 mg extracted for four hours in a micro-extractor with 80 % alcohol. The extract was transferred quantitatively to a 5-ml beaker, evaporated to dryness at 35° C under nitrogen, desiccated over calcium chloride under vacuum over night, and the residue then extracted with anhydrous ethyl ether. The ether-extracted residue was dissolved in 0.5 ml water, batch-deionized by adding 50 mg IRC-50 (H^+ form) and 100 mg IR4B (OH^- form) Amberlite exchange resins and shaken for two minutes, filtered through sintered glass into a 2-ml conical test tube, frozen over dry ice, and evaporated to dryness from the frozen state. The residue formed as a very light and fragile fluff during lyophilization,

[1] Received July 25, 1957.

[2] Paper No. 615 from the Department of Botany and Plant Pathology, Ohio State University, Columbus, Ohio.

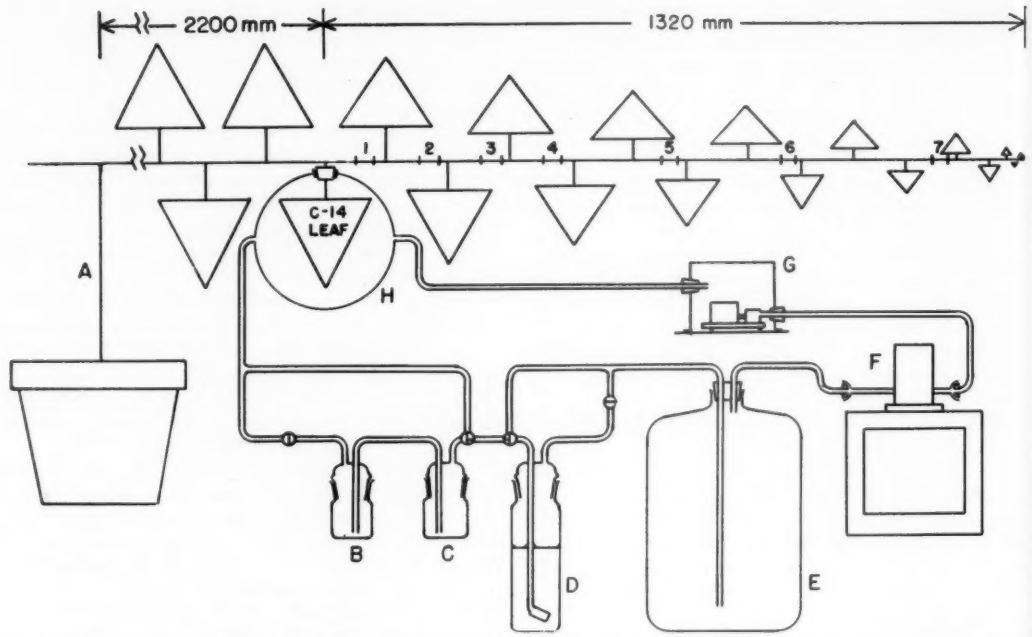

FIG. 1. Schematic drawing of apparatus for supplying $C^{14}O_2$ to single leaf on grape cane. A—grape vine bearing two canes (one shown); B—10 ml 17 % lactic acid; C—approximately 2 mc $BaC^{14}O_2$ plus 285 mg $BaCO_3$; D—absorption tube containing 10 % NaOH; E—50-liter bottle; F—ionization chamber with Brown recorder; G—diaphragm pump in leak proof housing; and H—3-liter Florence flask modified for leaf chamber. Nos. 1, 2, 3, etc. along cane indicate position of samples taken for analysis in expt. 1; in expt. 2, samples were taken near positions 2, 4, 5, and 7. Actual distances (center of sample section to node of C-14 leaf) for each experiment are given in tables I and IV respectively. (From Swanson, 16.)

and it was necessary to cap the test tube with a single layer of lens-cleaning tissue in order to prevent any loss from the test tube. Residue trapped by this filter could be readily dislodged back into the test tube by gentle tapping when lyophilization was complete. The residue was then dissolved in water and made up to a volume of 250 μl. This solution was then spotted on Whatman No. 1 filter paper for chromatographing.

Two chromatograms of each sample were prepared, one with a 50-μl aliquot (10 applications of 5 μl each, spaced 8 mm apart along the starting line) and the other with a 100-μl aliquot, prepared as above but double-spotted. The chromatograms were then developed by either continuous or multiple descent for 72 hours in *n*-butanol-ethanol-water (45 : 5 : 50 parts by volume). Guide strips of reference sugars were run near both margins, and at the completion of the run were cut off and sprayed with benzidinetrichloroacetic acid (1). The sugars were eluted from the chromatogram and quantitatively assayed by the anthrone method following essentially the procedure of Dimler et al (4). For radioactivity counts, 100-μl aliquots of each sugar were plated on stainless steel planchets.

At the start of each analysis, 200 μl (in later experiments, 250 μl) of a 0.05 % solution of rhamnose in 80 % alcohol was added to the pulverized plant material in the micro-extraction cup as an internal standard. The percentage recovery of the native sugars in the plant tissue was assumed to be the same as that of the added rhamnose. Rhamnose was chosen as an internal standard because it did not occur as a free sugar in the plant material used in these experiments and because its R_f value differs markedly from that of sucrose, glucose, and fructose. Blank corrections were obtained from the eluates of strips cut from the chromatogram in close proximity to the test strips, the anthrone-sensitive background of these strips ranging in value from approximately 0.50 μg to 0.85 μg sucrose-equivalent per cm^2 of filter paper. It is assumed that the preparative procedures employed prior to chromatographing the extracts eliminated most anthrone-sensitive substances capable of co-chromatographing with the various sugars.

RESULTS

EXPERIMENT 1. NORMAL CANE: Samples taken from the 1st, 2nd, 3rd, 4th, 6th, 8th, and 10th nodes acropetal to the C-14 leaf (fig 1) were analyzed. The distances from the node of the C-14 leaf to the center of each sample, and the respective concentrations of

TABLE I

CONCENTRATION OF SUGARS (LABELED + UNLABELED) IN THE STEM RELATIVE TO ACROPETAL DISTANCE FROM C-14 LEAF. EXPERIMENT 1

DISTANCE OF TRANSLOCATION (MM)	SUGARS AS % OF DRY WEIGHT					
	BARK			XYLEM *		
	SUC	GLU	FRU	SUC	GLU	FRU
88	5.75	2.81	1.24	2.65	3.71	2.03
202	5.16	2.52	1.86	3.05	3.94	2.01
321	6.22	3.01	1.04	3.29	4.23	2.72
429	5.94	3.05	0.84	3.08	4.46	2.49
652	6.44	2.79	1.13	3.48	5.68	2.62
875	6.27	1.71	0.59	3.23	6.49	2.84
1156	6.48	1.66	0.45	3.78	6.43	3.35

* Including the pith.

sucrose, glucose and fructose in both bark and xylem, calculated as a percentage of the dry weight of these tissues, are given in table I. It is evident from these data that appreciable quantities of all three of these sugars occurred in both the bark and xylem, sucrose being the conspicuously dominant sugar in the bark, and glucose, to a lesser degree, in the xylem.

Tables II and III provide the radiochemical data required for a more critical evaluation of the relative quantitative role of these sugars in translocation. These data, in addition to showing major radioactivity in the sucrose fraction in both bark and xylem, reveal that the relative concentrations of labeled-glucose and labeled-fructose, measured as counts per minute per milligram dry weight of tissue (columns 3 and 4 in tables II and III) were equal (if the reasonable assumption be permitted that the absolute specific activities of the glucose-C-14 and fructose-C-14 were identical, that is, that both were labeled to the same degree). This 1 : 1 equivalence in the radiochemical fractions of glucose and fructose suggests that the glucose-C-14 and fructose-C-14 in the stem were derived mainly by hydrolysis of the sucrose-C-14. On this basis, no significant translocation of the hexoses need be postulated to account for the occurrence or distribution of these sugars in the stem. Inasmuch as the bulk of the radioactivity on the chromatograms was recovered in the three sugar fractions, it may be inferred that sucrose is the only form of sugar which is rapidly translocated in the grape cane.

If this inference is correct, it may be predicted that the ratio of labeled-hexoses to labeled-sucrose will diminish with translocation distance in the phloem, in view of the decreasing time available for hydrolysis of the translocatory sucrose. It is evident from the data of table II that these predicted results were realized. Thus at a distance of 88 mm from the source leaf, the ratio of labeled-glucose (or -fructose) to labeled-sucrose was approximately 0.08, and at a distance of 1156 mm, the ratio had diminished to approximately 0.035.

Although the ratio of glucose-C-14 to fructose-C-14 was essentially unity, this relationship was not observed for the chemically determined fractions of these sugars. The latter fractions obviously represented pools composed mainly of older sugars in the cane which had undergone differential utilization. It should be noted in this connection that the radiochemical sugar fractions, on the other hand, represented recent increments in the stem, with an average age for the translocatory molecules and their derivatives of only about one hour, and it is reasonable to expect, therefore, that an insufficient time had elapsed for equilibration of the labeled sugar fractions with their respective sugar pools indigenous in the stem at the start of the experiment.

The data of table III refer to labeled sugars which accumulated in the xylem as a result of lateral movement from the phloem. The fact that longitudinal transport of C-14 labeled compounds in the xylem was negligible was established in an experiment in which the bark was separated from the xylem in the 2nd internode above the supply leaf. Although no severe restriction to translocation was anticipated by this treatment, inasmuch as the bark was not severed transversely, the damage to the phloem was greater than expected, perhaps mainly as a result of partial drying of the exposed phloem cells, and the separated-bark zone functioned essentially as a phloem-block.

TABLE II

RELATIVE CONCENTRATIONS OF C-14 LABELED SUGARS IN THE BARK AS A FUNCTION OF TRANSLOCATION DISTANCE. EXPERIMENT 1

DISTANCE OF TRANSLOCATION (MM)	CPM/MG DRY WT OF BARK			GLU/SUC	FRU/SUC
	SUC	GLU	FRU		
88	8005	661	678	0.083	0.085
202	6268	433	481	0.069	0.077
321	5800	397	402	0.069	0.069
429	4615	220	250	0.048	0.054
652	2942	136	126	0.046	0.043
875	1749	75	69	0.043	0.040
1156	900	34	31	0.037	0.034

TABLE III

RELATIVE CONCENTRATIONS OF C-14 LABELED SUGARS IN THE XYLEM AS A FUNCTION OF DISTANCE FROM C-14 LEAF. EXPERIMENT 1

DISTANCE OF TRANSLOCATION (MM)	CPM/MG DRY WT OF XYLEM *			GLU/SUC	FRU/SUC
	SUC	GLU	FRU		
88	1449	68	68	0.047	0.047
202	1182	63	63	0.053	0.053
321	903	58	72	0.064	0.080
429	523	61	59	0.116	0.113
652	404	38	35	0.094	0.086
875	202	29	28	0.144	0.139
1156	134	23	21	0.171	0.157

* Including the pith.

Although a small amount of labeled-carbon compounds moved past the treated zone, the greater portion of these undoubtedly moved in the phloem, and a conventional ringing experiment was regarded as unnecessary, therefore, to confirm the well established view that rapid translocation of photosynthate in the stems is primarily phloem-limited.

As previously remarked, the ratio of glucose-C-14 to fructose-C-14 in the xylem was approximately unity (table III), as in the bark; on the other hand, the ratio of labeled-hexoses to labeled-sucrose, unlike in the bark, increased rather than decreased with translocation distance from the source leaf. Thus, at a distance of 88 mm, the ratio of labeled-glucose (or -fructose) to labeled-sucrose was approximately 0.05, and at a distance of 1156 mm, approximately 0.16. Whether this reverse gradient resulted from fructose and glucose diffusing into the xylem and being carried in the transpiration stream, a possibility suggested by Nelson's data (13), or from a higher invertase activity in the younger xylem and pith cells near the tip, or from other factors, cannot be stated at present.

A comparison of tables I and III reveals that, with respect to the chemically-determined fractions of the several sugars in the xylem, glucose predominated by a relatively narrow margin, whereas with respect to the radioisotopic fractions, sucrose predominated by a relatively wide margin. In consideration of the respective age differences in the chemically-determined vs radioisotopically-determined sugar fractions, this observation further substantiates the important quantitative role of sucrose in translocation.

Experiment 2. "Single-Leaf" Cane. This experiment was carried out as described for the normal cane, with the exception that 48 hours prior to the start, all leaves were removed except the supply leaf. The purpose of the defoliation was two-fold: 1) to effect a reduction in the "native" sugar content in the stem, thereby providing a simpler background against which to interpret the superposed translocated sugars from the supply leaf, and 2) to eliminate the translocate supplied to the stem by any leaf other than the C-14 source leaf. Table IV presents the data obtained in this experiment. Apart from the expected reduction in sugar content of the bark (the xylem was not analyzed), the results are in reasonable agreement with those of the previous experiment. The change in ratio of the labeled-hexoses to labeled-sucrose in the bark with increasing distance from the supply leaf followed the same general trend as shown in the normal cane. The ratio of labeled-glucose to labeled-fructose approximated to unity, as required by the hypothesis outlined above, although the degree of fit was less satisfactory than in the previous experiment. It is possible that in a partially starved system, the translocatory sucrose and its derivatives would be metabolized in the phloem more rapidly than under more normal conditions, in which case equilibration with the existing pools of the respective sugars in the stem would be more rapid. Even allowing for this possibility, however, the agreement with a value of unity was within $\pm$ 9 %.

Table IV

Relative Concentrations of Labeled Sugars in the Bark as a Function of Translocation Distance. Experiment 2

Distance of translocation (mm)	cpm/mg dry wt of bark			Glu/Suc	Fru/Suc
	Suc	Glu	Fru		
172	15900	1390	1650	0.088	0.104
453	14200	800	860	0.056	0.061
650	11000	470	455	0.043	0.041
1022	10500	410	342	0.039	0.033

The data of both experiments strongly suggest, therefore, a high order of specificity for sucrose in the translocation process in grape.

Discussion

It is of interest to inquire now as to whether the apparently singular role of sucrose in translocation in grape may be a phenomenon of more general occurrence.

In recent years the sieve tube exudate of a considerable number of tree species has been analyzed chromatographically by various investigators (7, 19, 20, 21). In none of the species thus far studied (approximately 25) has either glucose or fructose been found to be present, even in chromatographically detectable traces, although sucrose was universally present. If the sieve elements are considered to be the primary conducting elements in the phloem tissue (15), then, in terms of the hypothesis that sucrose is the specific sugar of transport, it must be assumed that during transit a certain fraction of the sucrose molecules escape from the sieve tubes and are hydrolyzed in adjacent cells. Some corroboration of this view may be obtained from the fact that, although invertase appears to be absent in the sieve tube exudate of *Robinia pseudoacacia* (19), a positive test for this enzyme has been observed in whole tissue analyses of the phloem (8).

Zimmermann (21) has recently reported that, in addition to sucrose, several other sugars, namely raffinose, stachyose, and perhaps verbascose, may occur in the sieve tube exudate of certain species. In ash, appreciable concentrations of mannitol, a sugar alcohol, were also found. It appears likely, therefore, that sugars in addition to sucrose may serve as transport sugars in some species, but it is of interest to note, as pointed out by Zimmermann, that all of the true sugars thus far identified in sieve tube exudates are closely interrelated and include the sucrose moiety in their structure, differing from each other only in the number of included galactose residues. In about one-third of the species analyzed, only sucrose itself was present.

Additional evidence on the specificity of sucrose in

transport has been provided by Nelson (13), using a totally different approach. Solutions of various labeled sugars were supplied to soybean plants through cut petioles for short periods of time. Glucose and fructose moved rapidly past steam-girdled sections of the stem, as would be expected in typical xylem-injection experiments; sucrose translocation, however, was blocked, as would be expected in phloem-limited translocation. Although additional data are required before the divergent behavior of sucrose and the hexoses in these experiments can be explained, it may be tentatively inferred that the sucrose was rapidly accumulated in the phloem near the site of entry into the plant system, hence its transport could be blocked by killing the phloem. The evidence suggests, therefore, a specificity of sucrose in normal translocation.

Certain data in the literature suggest, however, a more significant role for glucose and fructose in translocation than has been indicated in the experiments discussed above. Dana (2), in a study of the physiology of dwarfing in apples, found that glucose was the primary compound in the bark containing radioactivity following a 30-minute period of C-14 photosynthesis in the terminal leaves of a branch. Vernon and Aronoff (18) concluded from their studies on soybean that sucrose, glucose, and fructose were all translocatory sugars, although the rate of translocation of the hexoses was less than that of sucrose. An alternative interpretation of these data, affirming the singular importance of sucrose, has been presented by Swanson (16), but the interpretation given by the authors remains a valid possibility. The present indications are, therefore, that the process of translocation in the apple and other species may well differ from that in grape in a number of important details. It is evident that a study of the comparative physiology of the sugar translocation mechanism in different species is greatly needed.

Summary

On the basis of radiochemical analyses, it was concluded that sucrose was the only form of sugar rapidly translocated in the grape cane. The C-14 label was introduced by supplying $C^{14}O_2$ to a single leaf on the cane under conditions favorable for photosynthesis. Appreciable quantities of labeled-glucose and -fructose were also found in the stem, but these appeared to be hydrolytic products of the translocatory sucrose, as inferred from the fact that the ratio of glucose-C-14 to fructose-C-14 approximated unity. These results accord with the data reported in the literature showing the complete absence of glucose and fructose, and the predominance of sucrose, in the sieve tubes of a considerable number of species (based on chromatographic analyses of the sieve tube exudate from these species). The convergence in results obtained by these disparate approaches provides supplementary evidence that the translocation of sugars occurs primarily in the sieve tubes or sieve cells of the phloem tissue.

We are indebted to Dr. N. J. Scully and Dr. John Skok for advice, and to Mr. J. William Burley for technical assistance. This investigation was supported in part by a research grant from the Rockefeller Foundation.

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CARBOHYDRATE AND ORGANIC ACID METABOLISM WITH C^{14} DISTRIBUTION AFFECTED BY COPPER IN THATCHER WHEAT[1]

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The participation of copper in plant and animal metabolism is well known (11), but its physiological role has not been defined in terms of distinct reactions. The role of copper in plant metabolism is complicated by the different copper requirements of plant species (5, 7). Data reported here are confined to observations made on Thatcher spring wheat, a plant with a comparatively high copper requirement.

In earlier work, Brown and Harmer (3) observed an almost immediate build-up of reducing sugars during stem elongation of copper-sufficient Thatcher wheat. This rapid increase of reducing sugars, not found in copper-deficient plants, seemed to indicate a marked change in metabolism as affected by copper. In view of this observation, attention in this study was given to the chromatographic determination of organic acids, sugars, and amino acids. The purpose was to determine relationships between sugar levels and the status of other metabolites in copper-sufficient and copper-deficient wheat.

It was reasoned that the change in sugars could be related to head development in that copper-sufficient wheat produces heads whereas copper-deficient wheat does not. To eliminate heading as a variable, copper-sufficient wheat grown on long nights was included in the study. Such plants do not produce heads.

MATERIALS AND METHODS

Thatcher spring wheat was grown on 544 g of organic soil which received 0.6 g KNO_3, 0.4 g $NH_4H_2PO_4$, 0.1 g $MnCl_2$, 0.5 g $ZnSO_4$, and 0.05 g H_3BO_3 per pot. Copper sulfate (0.15 g) was added to one half the pots. Twelve harvests were made of the short-night plants (6 hrs dark, 6 hrs light, 6 hrs dark, 6 hrs light), and 9 harvests of the long-night plants (12 hrs dark, 12 hrs light). The long-night plants were grown immediately following the short-night plants in the same light chamber at 75° F. Each light treatment was repeated by growing plants at the same temperatures in two different light chambers.

When radiocarbon was used, four pots, each containing 12 plants, were placed in a CO_2 chamber which was then partially evacuated. (In early harvests this number of plants was needed to furnish sufficient plant material.) Sulfuric acid was added to a CO_2 generator containing $BaC^{14}O_2$ and 3 mg $CaCO_3$ as a carrier, to generate enough $C^{14}O_2$ to introduce 50 μc of C^{14} into the chamber. Air, drawn through the generator and into the chamber, picked up the active CO_2 which was circulated by an inside fan turned by magnetic attraction to a rotating bar magnet.

Release of CO_2 from the soil in the pots made it impossible to control the CO_2 concentration inside the chamber. After two and one-half hours the pots were removed, the plants harvested and immediately analyzed.

In later harvests when plants were larger and sufficient material for analysis could be obtained from two pots, plants containing radiocarbon were allowed to remain 24 hours, passing through dark and light periods before harvest.

Ten g of fresh plant material were used for analysis and were immediately covered with hot 80 % ethanol, containing 1 g of $CaCO_3$. After boiling for two minutes the contents were transferred to a Waring blendor and blended for three minutes. The filtrate was separated from the residue by passing it through a No. 42 Whatman filter paper contained on a Buchner funnel. Filtrate volume was evaporated to 10 ml.

Two ml of this extract were placed in aluminum cups, evaporated to dryness, and counted in a gas flow counter. Eighteen lambda of the extract were placed on No. 1 Whatman filter paper and the amino acids chromatographed for 39 hours using butanol, acetic acid, and water (4 : 1 : 5) as the irrigant (8). Ninhydrin buffered with 2,4,6-trimethylpyridine was used to develop amino-acid spots on the chromatograph. Unknown amino acids were located on the chromatograph by comparing their location with that of known amino acids.

Sugars were chromatographed in the same way as the amino acids except that butanol, ethanol, and water (10 : 2 : 3) were used as the irrigant (13).

The remaining plant extract was used to determine reducing sugars quantitatively (12).

For organic acid analyses the $C^{14}O_2$ treatment and harvest of plant materials were identical and coincident with those mentioned above. Plants were oven dried at 70° C, ground to 20 mesh, mixed with 1.5 g of acid-washed asbestos, and acidified to pH 1. Organic acids were extracted from 0.75 g dry plant material with ethyl ether. Extraction continued 48 hours in Soxhlet extractors. Three ml of 0.1 *N* NaOH were added to the extract which was heated with frequent stirring on a warm steam bath to remove the ether. The resulting solution containing the organic acids was transferred to four discs cut from botanical drying paper. These discs were placed in a column containing silica gel and the acids chromatographed (1).

Three-ml aliquots were collected and titrated with 0.01 *N* NaOH to locate the acids. After titration, the

[1] Received August 1, 1957.

aliquots were placed in aluminum cups, evaporated to dryness and the C^{14} activity determined by counting in a gas-flow counter.

Results

Wheat grown on long nights (12 hrs dark, 12 hrs light) with copper failed to head (fig 1). Grown on short nights (6 hrs dark, 6 hrs light, 6 hrs dark, 6 hrs light), heads developed on copper-sufficient plants (fig 1). Internode elongation was greatest in the copper-sufficient, short-night plants and least in the copper-deficient, short-night plants. Internodes did elongate somewhat in the copper-sufficient, long-night plants (fig 1).

During internode elongation there was a sudden accumulation of reducing sugars in both short- (table I) and long-night plants (table II). This occurred when the plants were about 46 days old, before heads developed. The 80 % ethanol-extractable C^{14} tagged metabolites (water soluble) nearly doubled in the copper-sufficient as compared with the copper-deficient plants (tables I and II). When harvest was delayed 24 hours after exposure to $C^{14}O_2$, the 80 % ethanol-extracted C^{14} ratio of copper-sufficient to copper-deficient increased from 2 to 6 for short-night plants, but remained approximately two for long-night plants.

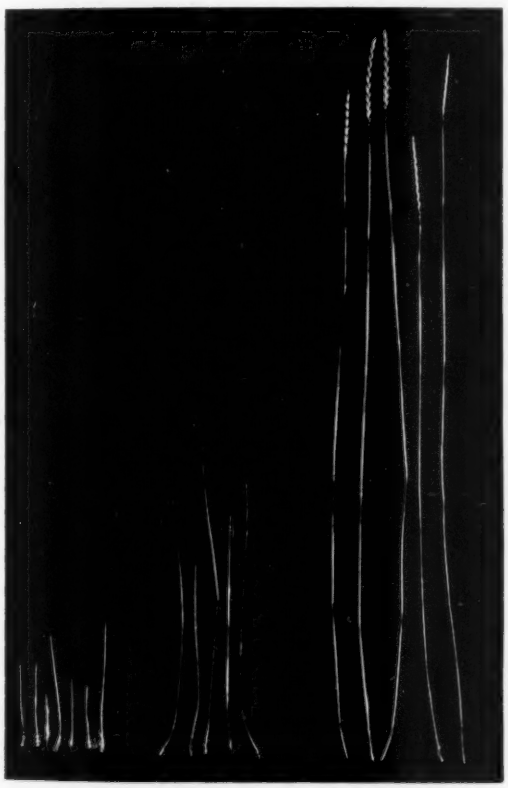

Fig. 1. Effect of copper and length of night on head development in Thatcher spring wheat. Left to right; copper-deficient wheat (short night), copper-sufficient wheat (long night) and copper-sufficient wheat (short night).

Table I

Yield of C^{14} * and Percent Reducing Sugar of Cu-Sufficient and -Deficient Thatcher Spring Wheat—Short Night **

Age of plants	Yield dry wt/plant		C^{14} ratio Cu/No Cu	Reducing sugar (dry wt)	
	No Cu	Cu		No Cu	Cu
days	*g*	*g*		%	%
15	.04	.04	1.01	.50	.46
19	.07	.07	.98	.62	.79
21	.07	.07	1.00	.65	.63
23	.06	.08	1.11	.71	.73
28	.12	.16	1.14	1.04	1.16
33	.18	.27	1.01	.51	.59
35	.20	.33	1.22	.58	.66
39	.24	.40	1.16	.78	1.50
42	.24	.44	1.04	.44	1.06
46	.27	.70	2.03	.79	7.31
47	.32 ***	.69 ***	6.08 ***	1.06 ***	6.24 ***
50	.30	.70	2.09	.61	4.97

* 80 % ethanol extractable (water soluble).
** Short night = 6 hrs light, 6 hrs dark, 6 hrs light, 6 hrs dark.
*** Harvested 24 hours after exposure to $C^{14}O_2$ instead of 2.5 hours as in all other treatments.

Radioautographs of chromatographs (fig 2) show the C^{14} located in the sugar spots of short-night plants. The light strip is the chromatograph, with the dark spot showing a positive test for sugar. The dark strip is the radioautograph of the chromatograph with the darker areas showing the presence of C^{14} Only one sugar spot was detected in the 21-day-old

Table II

Yield of C^{14} * and Percent Reducing Sugar of Cu-Sufficient and -Deficient Thatcher Spring Wheat—Long Night **

Age of plants	Yield dry wt/plant		C^{14} ratio Cu/No Cu	Reducing sugar (dry wt)	
	No Cu	Cu		No Cu	Cu
days	*g*	*g*		%	%
20	.05	.07	1.01	.62	.62
25	.10	.09	1.23	.60	.88
27	.10	.13	1.14	.46	.48
33	.14	.26	1.08	.58	.49
35	.18	.25	1.21	.52	.46
39	.23	.35	1.16	.70	1.43
41	.22	.40	1.20	.67	1.06
46	.27	.57	2.12	.73	5.77
47 ***	.21 ***	.54 ***	2.37 ***	.85 ***	4.42 ***

* 80 % ethanol extractable (water soluble).
** Long night = 12 hrs light, 12 hrs dark.
*** Harvested 24 hours after exposure to $C^{14}O_2$ instead of 2.5 hours as in all other treatments.

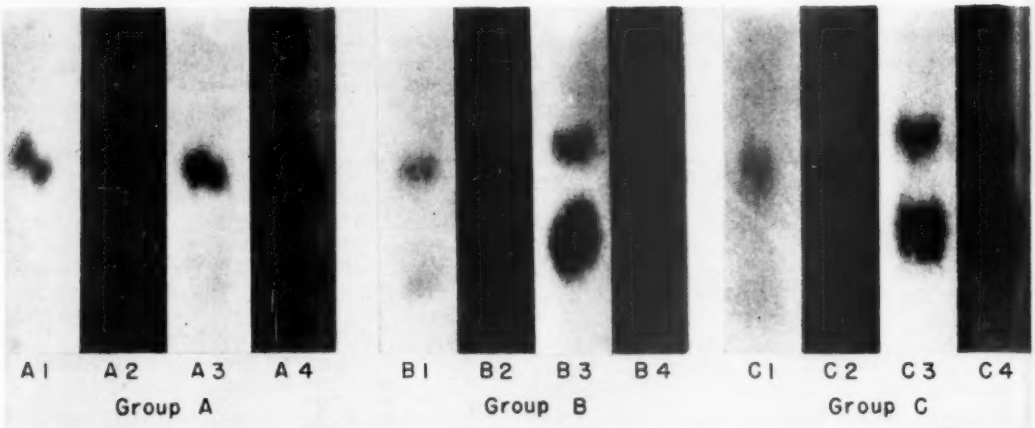

FIG. 2. Effect of copper on sudden build-up of sugars in Thatcher spring wheat grown with short nights. Light strips are chromatographs with dark spot showing positive test for sugar. Dark strips are radioautographs of chromatographs with dark spots showing presence of C^{14}. Note one sugar spot in 21-day-old plants and two spots in the 46-day-old plants.

Group A—21-day-old plants harvested 2.5 hours after exposure to $C^{14}O_2$.
Group B—46-day-old plants harvested 2.5 hours after exposure to $C^{14}O_2$.
Group C—46-day-old plants harvested 24 hours after exposure to $C^{14}O_2$.

copper-deficient (A 1) and copper-sufficient (A 3) plants.

When the plants were 46 days old, two sugar spots were evident on the chromatographs (fig 2, B 1, B 3). The first spot matched that of sucrose and the second spot that of the C_6 sugars when compared with chromatographs of known sugars. The chromatographs (B 1, B 3) indicate much less sugar in the copper-deficient (B 1) than in the copper-sufficient plants (B 3). This is especially true of the C_6 sugars. The radioautographs of the chromatographs show less C^{14} in the copper-deficient sugar spot (B 2) than in the copper-sufficient spot (B 4).

The chromatographs and radioautographs in figure 2, groups B and C, represent plant materials exposed to $C^{14}O_2$ at the same time for two and one-half hours. Plant materials in group B were harvested immediately. Plant materials in group C were harvested 24 hours later. Copper-sufficient plants (B 3, B 4, C 3, C 4) indicate much more C^{14} and sugars, especially C_6 sugars, than copper-deficient plants (B 1, B 2, C 1, C 2). The 24-hour delay of harvest reveals a noticeable decrease of C^{14} activity in the sugar spots of copper-sufficient plants (C 4 compared to B 4), and an almost complete disappearance of C^{14} activity in sugar spots from copper-deficient plants (C 2 compared to B 2).

Organic acid concentration decreased in the copper-sufficient plant material when the sugar concentration increased. Figure 3 shows the chromatographic separation and C^{14} activity of organic acids in short-night plants 42 days old (9-13-56) and 50 days old (9-21-56). It is evident that the organic acid concentration in copper-sufficient Thatcher wheat changed suddenly during this period of growth. This change was not observed in the copper-deficient plants. Citric acid fractions (150 to 160) showed the first evidence of any change.

The greatest C^{14} activity occurred in fractions (123 to 127) which are believed to contain malic acid (fig 3). This activity was about the same for 42-day-old copper-sufficient and -deficient plants, but decreased to practically no activity in the copper-sufficient 50-day-old plants. Copper-deficient plants retained considerable activity in these fractions. Figure 4 shows a comparative disappearance of C^{14} activity from the organic acid fractions of copper-sufficient and -deficient wheat during a 24-hour period. The concentration of acid remained about the same, but the C^{14} activity in the copper-deficient plants had markedly decreased. Such data indicate a relatively rapid conversion of the organic acids in copper-deficient wheat.

Amino acid concentrations in copper-sufficient wheat did not change as much as concentrations of sugars and organic acids. Changes did occur in copper-deficient wheat as the deficiency symptoms became evident. Asparagine and aspartic acid began to appear in greater concentrations. The amino acids continued to increase in concentration with increasing copper deficiency. Copper-sufficient plants showed a relatively small accumulation of asparagine and aspartic acid and contained the same, but lesser quantities of the other amino acids.

DISCUSSION

Copper requirements are not the same for all plant species. Copper-deficiency symptoms become most evident as the plant passes from a vegetative to a reproductive stage of growth (5). This is true for

wheat, oats, corn, and cocklebur. The effect of copper on some of the end-products of metabolism in Thatcher spring wheat is illustrated by sudden changes in the concentration of sugars and organic acids in copper-sufficient plants. These changes are very marked, but they are believed to result from more gradual changes which have taken place during the maturing of the plant.

The evidences for these changes in Thatcher wheat which denote a difference between copper-deficient and copper-sufficient plants are:

1. An early loss of turgidity in leaves of copper-deficient plants.
2. Increased concentration of chlorophyll during early growth of copper-sufficient plants; no increase in copper-deficient plants (2).
3. Dieback from tips of copper-deficient leaves when internode elongation takes place. This does not occur in copper-sufficient plants (2).
4. Internode elongation is much more pronounced in copper-sufficient plants.
5. Copper-sufficient plants develop normal heads; copper-deficient plants do not.

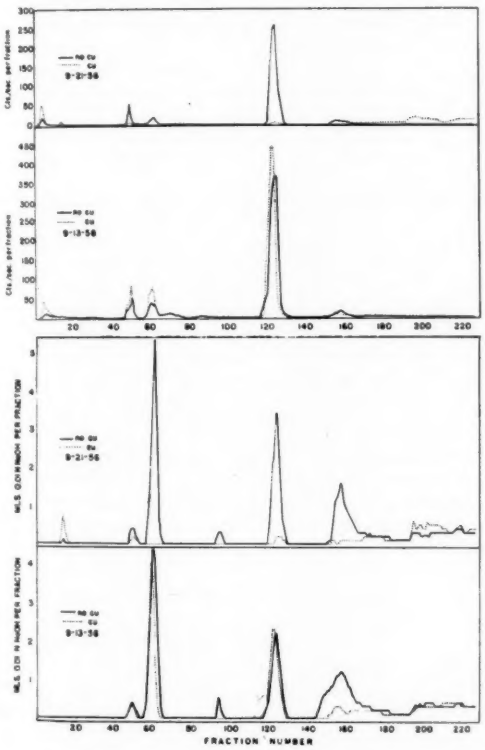

FIG. 3. Chromatographic comparison of organic acid fractions with C^{14} in the fractions of copper-deficient and copper-sufficient Thatcher spring wheat at two harvest dates. Note decrease in acids and count (9-21-56) of copper-sufficient plants.

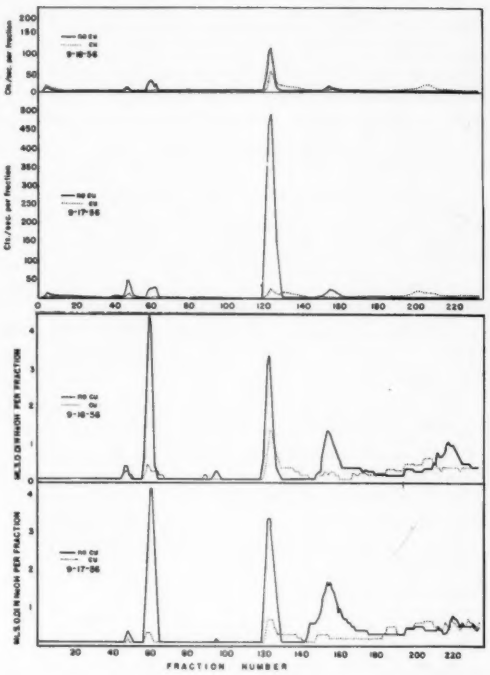

FIG. 4. Chromatographic comparison of organic acid fractions with C^{14} in the fractions of copper-deficient and copper-sufficient Thatcher spring wheat harvested 2.5 hours after exposure to $C^{14}O_2$ (9-17-56) and 24 hours later (9-18-56). Note turnover of C^{14} in copper-deficient plants during a 24-hour period.

6. Nitrogen (including nitrates), phosphorus, and potassium remain at relatively high concentrations in copper-deficient wheat; these concentrations are much lower in copper-sufficient plants (2). The latter may be a dilution effect related to greater yields.
7. Copper-deficient wheat shows a reduced ascorbic acid oxidase activity in the early stages of growth, as compared with copper-sufficient wheat (4).
8. More of the dye, 2,3,5-triphenyltetrazolium chloride is reduced in the nodal areas of copper-sufficient than copper-deficient wheat (6).

Copper affects the internal reducing capacity of the corn plant (6). Copper-deficient corn did not reduce the dye, 2,3,5-triphenyltetrazolium chloride, but the dye was reduced in copper-sufficient corn. Catalase activity was higher in copper-deficient corn, tobacco, and tomatoes than copper-sufficient plants (5, 4).

Copper is one of the elements that may occur in metallo-flavoproteins (9, 10). As such, it may link areas of hydrogen transfer during metabolism to those of electron transport proper.

Internally there appears to develop a changed redox level in copper-sufficient as compared with copper-deficient wheat plants. There is a change in oxidative enzymes and reducing capacity which may affect a variable accumulation of substrates. The rapid shift in organic acids and sugars of copper-sufficient plants, not found in copper-deficient plants, reflects a possible altered rate or change in metabolism.

Organic acids occupy a central position in the metabolism of plants. They are early products of photosynthesis and as such serve as precursors for the synthesis of many other compounds. The close metabolic relationship of organic acids to fats, carbohydrates, and proteins emphasizes their key role in plants and reflects the important role of copper in determining the status of these compounds in Thatcher wheat.

To understand the causative factors involved in the shift in concentration of organic acids and carbohydrates of copper-sufficient wheat would require an understanding of the role of copper in the nutrition of wheat in terms of distinct reactions. Results from these studies give a basis for studying specific reactions which may more closely define the effect of copper in these metabolic processes. These reactions remain as a challenge for future work.

Summary

Organic acids, sugars, and amino acids were determined chromatographically in Thatcher spring wheat grown under short and long nights. There was a marked decrease in organic acids and a simultaneously large increase in sugars of 46-day-old copper-sufficient plants. Distribution of C^{14} from $C^{14}O_2$ increased in accordance with the above products formed. Copper-deficient plants did not show these changes.

Copper-sufficient plants accumulated less asparagine and aspartic acid than copper-deficient plants and comparably contained the same, but lesser quantities of other amino acids.

The effects of copper on these end-products of metabolism are illustrated and discussed with reference to other changes which occur in copper-deficient wheat. The changed sugar and organic acid metabolism was not directly related to head development.

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BETA-ALANINE PROTECTION OF YEAST GROWTH AGAINST THE INHIBITORY ACTION OF SEVERAL CHLORINATED ALIPHATIC ACID HERBICIDES[1]

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Chloro-substituted acetic, propionic, and isobutyric acids constitute a class of compounds which in recent years have demonstrated considerable potential use for the control of a number of weedy grasses in several weed-crop situations. The structural similarity of these compounds to propionic and acetic acids makes it seem probable that their phytotoxicity might result, in part, from interference with pantothenic acid synthesis. Such an effect has been demonstrated with the two unchlorinated acids by King and Cheldelin (1) using yeast as a test organism. Their results led to the conclusion that "propionate inhibits growth by competing with β-alanine for attachment within the yeast cell, thereby preventing the coupling of the pantothenic acid moieties."

In the experiments reported herein, an investigation has been made of the interactions of β-alanine and several chlorinated aliphatic acid herbicides on the growth of yeast using the technique of King and Cheldelin.

Saccharomyces cerevisiae, isolated from the center of a cake of baker's yeast (Fleischmann's) was inoculated onto 2 % agar nutrient medium containing the same major inorganic salts as used by King and Cheldelin (1) but with 2.5 g yeast extract (Difco) in lieu of their minor elements and vitamin supplements. A liquid subculture was prepared for each experiment in a 125-ml flask containing 30 ml of liquid medium (1) to which was added 100 μg β-alanine in a 10-ml volume of water. The flask was inoculated with yeast cells from an agar slant and incubated for 24 hours at 32° C. The resulting culture was diluted 1:50 with nutrient solution to constitute the experimental inoculum. Growth experiments were carried out under aseptic conditions in 18 × 150-mm Pyrex test tubes. Each tube contained 5 ml of pH 4.8 nutrient, and 1 ml each of β-alanine solution, herbicide solution and diluted liquid subculture of yeast. After 17 hours of incubation at 32° C, turbidimetric growth readings were taken in a Klett-Summerson colorimeter using a blue filter. All experiments were run in triplicate and repeated with three or more cultures.

In the absence of exogenous β-alanine, turbidity readings of 40 to 60 but occasionally as high as 120 Klett-Summerson units were obtained. Addition of 0.2 to 200 μg of β-alanine per tube increased growth to 160 to 180 units. The minimum amount of metabolite required for maximum growth was generally 0.2 μg per tube, and 200 μg appeared to be at least a 10-fold excess for maximum protection against herbicides. These two concentrations of β-alanine were consequently selected for use throughout the present studies.

The herbicidal chemicals,[2] obtained as purified acids, were dissolved in sterile water and the solutions were adjusted to the pH of the medium with sterile NaOH. These solutions were added aseptically to the sterile culture tubes immediately before inoculation. The acids used in these studies, their abbreviated designations, and purities were as follows: 2,3,3′-trichloroisobutyric acid (TCIB), 90 % [impurities of 2,3,3-trichloroisobutyric and 2,3-dichloroisobutyric acids]; 2,3-dichloroisobutyric acid (DCIB), chemically pure; 2,2,3-trichloropropionic acid (TPA), 99+ %; 2,2-dichloropropionic acid (dalapon), 99+ %; and trichloroacetic acid (TCA), USP.

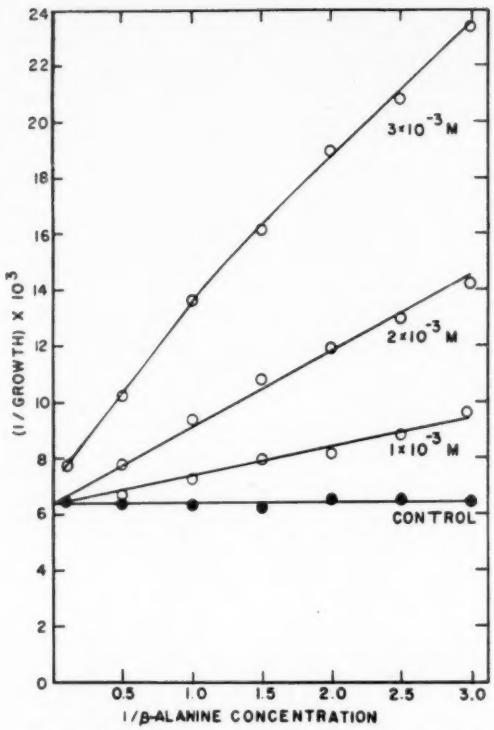
Evidence was obtained for protection by β-alanine against each of the five herbicides tested. The most definite suggestion of competition between herbicide and metabolite was observed with the chlorinated isobutyric acids. A positive test for a competitive relationship between TCIB and β-alanine (fig 1) was obtained in an analysis of data by the method of Lineweaver and Burk (2). Pantothenic acid was also found to be effective as an antagonist of TCIB.

In comparative studies TCIB was found to be slightly more inhibitory than DCIB. In the presence of 0.2 μg of β-alanine TCIB caused 50 % inhibition at 3 to 6×10^{-4} M, while ca. 10^{-3} M DCIB was required to produce the same degree of inhibition. At the higher metabolite concentrations (200 μg of β-alanine or 10 μg calcium pantothenate), TCIB at 5 to 8 $\times 10^{-3}$ M reduced growth by 50 % and at 10^{-2} M generally gave complete inhibition. DCIB, however, at concentrations below 10^{-2} M consistently caused less than 25 % inhibition in the presence of the higher concentration of β-alanine. At its high concentrations, therefore, β-alanine more readily overcame the inhibitions caused by DCIB than those produced by TCIB.

Evidence for a protective action by β-alanine against the growth inhibitory actions of unchlorinated isobutyric and *n*-butyric acids was observed with some cultures; however, results between experiments were

[1] Received August 13, 1957.

[2] The experimental compounds used in these studies were supplied by research personnel of the following companies: American Chemical Paint Company, Ambler, Pa.; American Cyanamid Company, Stamford, Conn.; Dow Chemical Company, Midland, Michigan; Rohm and Haas Company, Philadelphia, Pa.



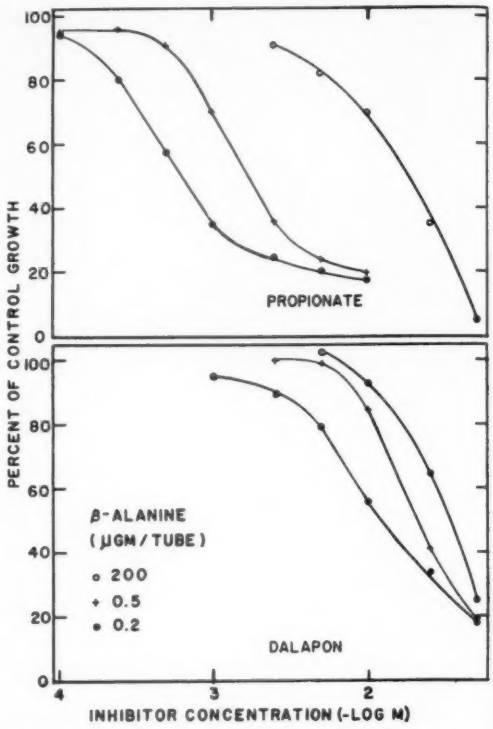


FIG. 1 (*left*). Competitive inhibition test for β-alanine and TCIB. Yeast growth measured in arbitrary turbidity units. Concentration of β-alanine expressed as µg per 8 ml of solution.

FIG. 2 (*right*). Comparison of inhibition of yeast growth by propionate and dalapon at three concentrations of β-alanine. Concentration of β-alanine expressed as µg per 8 ml of solution.

too variable to justify quantitative comparisons. The variability appeared to reside in differential requirements of cultures for an exogenous supply of β-alanine. In no instance did the unchlorinated compounds inhibit growth at concentrations as low as those used for the chlorinated isobutyrates.

A recently procured sample of dalapon, 99+ % pure, was compared directly with propionic acid, as shown in figure 2. Although β-alanine offered some protection against this herbicide, the effect was not as pronounced as that observed with the unchlorinated parent acid. Chlorination of propionic acid obviously decreased the ability of the molecule to compete with β-alanine. A 2nd sample of dalapon which was studied had been obtained as the purified acid and allowed to age on the shelf for three years. This sample, which had apparently undergone some decomposition at the time of use, gave a positive competitive inhibition test by the Lineweaver-Burk method and closely approached the inhibitory effectiveness of propionic acid. Although this sample was more inhibitory to growth of yeast than the undeteriorated sample, a similar increase in phytotoxic activity could not be demonstrated against higher plants. Foliar spray application of the deteriorated sample to 8 crop and weed species in the greenhouse showed no evidence that decomposition had in any way altered the herbicidal properties of the sample.

Relatively high concentrations of the trichloropropionic and trichloroacetic acids were required for inhibition of yeast growth. Only slight protection by β-alanine was observed against inhibitor concentrations of 10^{-2} to 10^{-1} M. Data are shown in table I for a common concentration of TPA, TCA, and acetic acid. At low β-alanine concentrations the unchlori-

TABLE I

BETA-ALANINE ANTAGONISM OF INHIBITORS OF YEAST GROWTH

INHIBITOR (0.05 M)	PERCENTAGE INHIBITION IN VARIOUS CONCENTRATIONS * OF BETA-ALANINE		
	0.2 µG	2 µG	200 µG
2,2,3-Trichloropropionic acid	40	..	19
Trichloroacetic acid	36	20	19
Acetic acid	61	0	6

* Amounts in 8 ml of solution.

nated acetic acid was more inhibitory than TCA and was more readily antagonized by β-alanine.

The inability of high concentrations of β-alanine completely to reverse the inhibitions produced by this class of compounds suggest that physical or chemical processes other than the β-alanine → pantothenic acid reaction(s) may be involved in the mechanisms of action. Nevertheless, the utilization of β-alanine appears to be the most sensitive metabolic process involved under the experimental conditions employed; the enzymes involved in pantothenic acid synthesis should be investigated as a possible important site of action of these herbicidal compounds. Further investigations on this mechanism of action and its relative importance in species selectivity are in progress and will be the subject of future reports from this laboratory.

Summary

Inhibition of yeast growth by chloro-substituted isobutyric, propionic, and acetic acids has been observed. An exogenous supply of β-alanine has been shown to produce varying degrees of protection against these inhibitions.

The results obtained suggest that these herbicides interfere with synthesis of pantothenic acid, presumably by competition with β-alanine.

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A PAPER CHROMATOGRAPHIC SEPARATION OF GIBBERELLIC ACID AND GIBBERELLIN A[1]

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Gibberellin A and gibberellic acid have been separated on buffered partition columns by Stodola et al (1). We now report a paper chromatographic method for separation of these plant growth stimulators.

Takahashi et al (2) reported the use of the upper phase from various mixtures of benzene, acetic acid, and water for paper chromatography of a preparation of gibberellin A using BPB (which we assume to be bromphenol blue) as an indicator. They reported gibberellin A to be immobile in all these mixtures. Phinney et al (3) using four different solvent systems reported that they were unable to separate the gibberellins in any of the four systems.

We have found that the upper phase of a mixture of 10 vol of thiophene-free benzene, 2.5 vol of glacial acetic acid, and 5 vol of deionized water will completely separate gibberellin A and gibberellic acid on a descending chromatogram under conditions which permit sufficient solvent flow.

Samples containing 50 to 200 μg of the gibberellins are applied near one end of a sheet (19 × 46 cm) of Schleicher and Schuell No. 598 or Munktells, Cremer-Tiselius electrophoretic filter paper. The opposite end of the sheet is serrated to permit solvent to drip off the end more freely, thus allowing a maximum flow. A 10- to 30-minute equilibration of the spotted paper and a descending development of at least 36 hours at 22 to 24° C are carried out in a glass jar (12 × 24 in). The jar is fitted with a rack holding a 300-ml stainless steel trough and with a glass lid having a hole for addition of solvent. The inside wall of the jar is lined with a filter paper cylinder, the bottom edge of which is immersed in the lower phase of the solvent mixture in the bottom of the jar. The liner should be allowed to become completely wet before using the jar for chromatography. Once prepared, a jar can be used many times.

After development the chromatogram is removed from the jar and is dried at room temperature. When dry, it is sprayed lightly with a 0.5 % aqueous solution of potassium permanganate. Gibberellin A and gibberellic acid are revealed as yellow spots on a reddish-purple background. The spots are quickly sprayed with more permanganate and the chromatogram is immediately washed in running tap water to remove the unreacted permanganate. The locations of the gibberellins are detected as permanent brown spots on an almost white background. Figure 1 is a photograph of a typical chromatogram treated in the above manner showing the separation of the gibberellins achieved with this solvent system.

Gibberellic acid may also be detected on chromatograms not reacted with permanganate, or sprayed only very lightly with permanganate, by its reaction with sulfuric acid to produce a fluorescence under ultraviolet light. This may be done with concentrated sulfuric acid as reported by Phinney et al (3) or by rapidly dipping the paper in 70 % sulfuric acid and quickly laying it flat on a glass plate. A chromatogram treated with the 70 % sulfuric acid shows yellow-green fluorescent spots instead of the blue fluo-

[1] Received August 27, 1957.

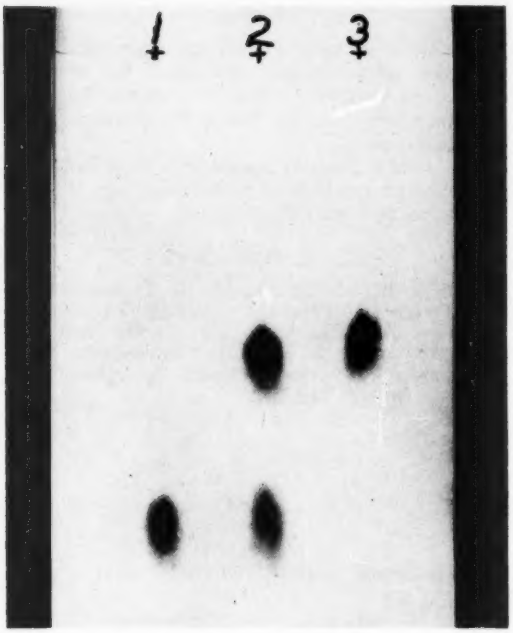

FIG. 1. Photograph of a chromatogram: 1, Gibberellin A (Stodola); 2, Mixture of gibberellin A (Stodola) and gibberellic acid; 3, Gibberellic acid. The chromatogram was developed on Munktells, Cremer-Tiselius electrophoretic paper for 46 hrs at 22° C. 100 μg of each of the gibberellins were used.

rescence produced by concentrated sulfuric acid. We have found the 70% sulfuric acid treatment preferable since it allows easier handling because the paper disintegrates more slowly. The fluorescent spots may be photographed for a permanent record.

This paper chromatographic system is useful for the qualitative analysis of purified and partially purified preparations of the gibberellins. It satisfies the requirement for a relatively quick, easy method for detection of gibberellin A in gibberellic acid preparations. This has not been possible with the solvents previously reported. The procedure described may be useful for more complete identification of gibberellin-like compounds found in plant extracts.

We wish to thank Dr. F. H. Stodola, N.U.R.B., USDA, Peoria, Illinois, for the sample of purified gibberellin A used for this work.

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GROWTH OF ETIOLATED SECTIONS OF PEA INTERNODE FOLLOWING EXPOSURES TO INDOLE-3-ACETIC ACID, 2,4-DICHLOROPHENOXYACETIC ACID AND 2,5-DICHLOROBENZOIC ACID[1]

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A striking feature in the assessment of plant growth regulating substances is the discrepancy between results obtained with isolated tissues and whole plants. For instance, indole-3-acetic acid is considered to be more physiologically active than 2,4-dichlorophenoxyacetic acid in most of the standard laboratory tests on excised tissues, but when applied to whole plants it shows low activity, and although a rapid initial epinastic response may occur, it does not normally cause the long-term formative distortions so typical of 2,4-dichlorophenoxyacetic acid and allied herbicidal substances. There is considerable evidence to suggest that the stability of a compound within the plant and the ease with which it can penetrate and be transported contribute to this difference in response. But a further factor might be considered, namely the variation in the length of time the tissues remain in contact with the growth substance.

In laboratory investigations, rapidly elongating sections of stem or coleoptile are most frequently used, and the sections are normally exposed to a known concentration of the growth substance for the whole experimental period. However, when these chemicals are applied to whole plants, only one dose of the growth regulator is usually given and it is the growth response subsequent to this application which is studied. Comparable studies in short-term laboratory tests have received scant attention, although some investigations have been carried out with roots (8, 12). Apart from experiments with supraoptimal concentra-

[1] Received September 6, 1957.

tions on Avena coleoptile sections (9) little is known about the growth responses of excised sections of stem tissue following a single brief period of exposure to growth substances. Experiments were therefore planned to determine the effect upon straight growth of short exposures to suboptimal doses of a number of well known growth regulating substances, each of different chemical structure. Sections of etiolated pea epicotyl were supplied with the compounds for a few hours only. The extension growth was studied when the sections were transferred either to water or to further low concentrations of active growth substances.

By these means clear differences in growth response have been demonstrated between sections which have been treated with indole-3-acetic acid (IAA) and those treated with 2,4-dichlorophenoxyacetic (2,4-D) or 2,5-dichlorobenzoic acids (2,5-DBA).

Material and Methods

Choice of Method: Considerable attention was initially devoted to finding a convenient, reliable and rapid method of assaying growth in sections of plant material. Determinations of the increase in length of sections cut from the elongating portion of stem or coleoptile have been favored by most workers, but since the length of section obtainable from the region is small (usually 1 cm), the growth increments for periods of less than several hours are difficult to measure with great accuracy since they too are small and the measurement is subject to considerable visual error.

Experiments showed that etiolated plants of *Pisum sativum* of considerable uniformity can readily be grown and that a satisfactory elongation response can be obtained from 1-cm sections of the epicotyl when they are grown in aqueous solutions of the sodium salts of IAA, 2,4-D or 2,5-DBA without the addition of sugars or buffers. If the volume of ambient solution is large (40 ml/10 sections) no pH changes are detectable in the external solution during the experiment (24 hours). A simple determination of weight made on a torsion balance is subject to a considerably smaller error than that involved in the measurement of the length of a section, since no correction need be applied for curvature, or other distortion of the section during growth, or for cut ends which are not exactly transverse. Further, the rapidity with which such weight determinations can be made, renders it possible to repeat the operation at frequent intervals with little damage to the tissue.

The increase in length of segments growing in suboptimal solutions of IAA (0.01 to 5.0 ppm), 2,4-D (0.005 to 2.5 ppm) and 2,5-DBA (0.05 to 15.0 ppm) was found to be directly proportional to the increase in fresh weight of the tissues for any test period up to 24 hours from the start of an experiment. Therefore, the conclusions drawn here from measurements of fresh weight are equally applicable to extension growth.

Method: Peas, var. Alaska, were soaked for several hours in distilled water at 24.5° C, then sown in enamel troughs of sterile washed sand. The troughs were kept in a controlled conditioned dark room at 24.5° C and watered with standard volumes of distilled water. Periods of 10 to 15 minutes of red light (Wratten O with 40-watt bulb) were given daily, once the shoots had emerged above the surface. Under these conditions, the 3rd internode was 2 to 3 cm long on the 7th day from planting, and the plants were ready for use. One section, 1 cm long, was cut from the 3rd internode, just below the crook. Ten sections were bulked, weighed on a torsion balance, and then

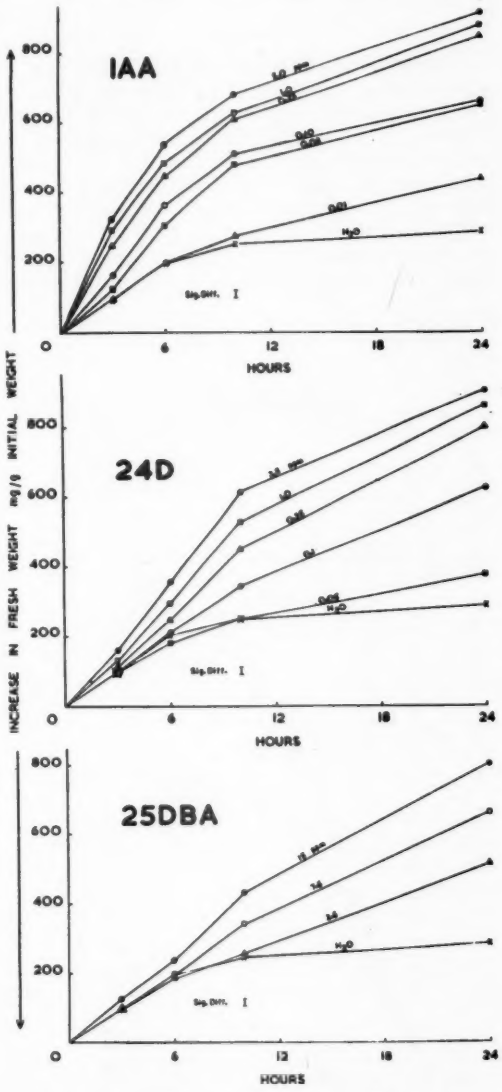

Fig. 1. Extension growth of sections of etiolated pea internode grown for 24 hours in solutions of IAA, 2,4-D or 2,5-DBA, expressed as the increase in fresh weight mg/g initial fresh weight.

floated upon the surface of the operative solution, the volume of which was not less than 40 ml. When the sections were next weighed, they were transferred from the solution to clean blotting paper and the surface liquid gently removed. Tests in which sections were immersed, blotted and weighed several times in succession have shown that the error due to differences in the removal of surface water are in the order of 0.5 to 1 %. Each treatment was duplicated. The technique can be recommended as a useful method for determining small increments of growth.

Results

Growth of Sections of Pea Internode Kept in Solutions of IAA, 2,4-D or 2,5-DBA for 24 Hours: The sections were grown in a range of concentrations of IAA, 2,4-D and 2,5-DBA. Measurements of the fresh weight were made at intervals of 3, 6, 10 and 24 hours from the start of the experiment and the sections were transferred to fresh solution after each weighing. The curves obtained for each compound are shown in figure 1. At these suboptimal concentrations the growth increments of the treated sections are either greater than or approximate to but are never significantly less than that of the controls.

Growth of Sections of Pea Internode Treated with IAA, 2,4-D or 2,5-DBA for Varying Periods and then Transferred to Water: Sections were floated on solutions containing a range of concentrations of growth regulators for periods varying from one to three hours, after which they were removed from the solution, blotted and weighed again. They were rinsed and transferred to dishes of water, and the water changed after each subsequent weighing up to 24 hours from the start of the experiment. A selection of the results is presented in figures 2, 3 and 4. It was ascertained that very little further increase in fresh weight ever occurred after 24 hours in any of the experiments described.

The results for IAA treatments of 1 to 25 ppm for one hour are shown in figure 2 A and 2 B. The rate of growth of some of the treatments eventually falls below that of the control. This is first apparent in sections receiving the smallest dose of IAA (1.0 ppm) and becomes evident somewhat later in those receiving larger doses (5.0, 12.5 and 25 ppm). The rate

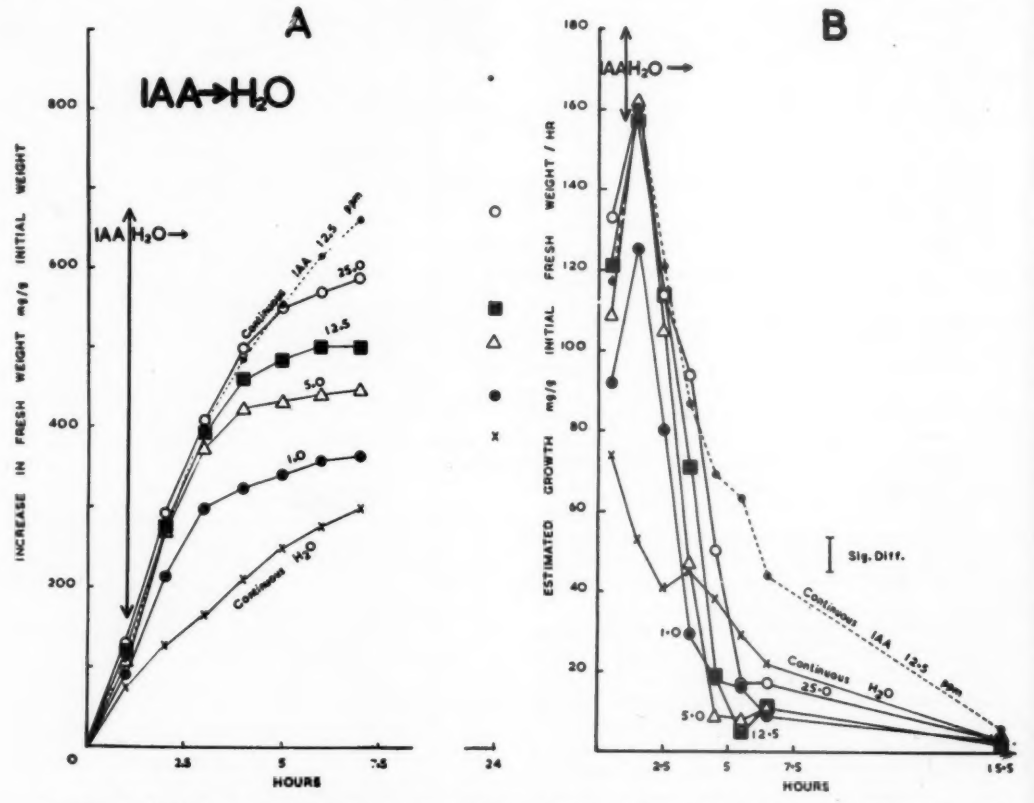

Fig. 2. A. Extension growth of sections of etiolated pea internode treated for 1 hour with different concentrations of IAA followed by 23 hours in H_2O, expressed as increase in fresh weight mg/g initial fresh weight.

B. The data from 2 A plotted as the estimated growth in mg/g initial fresh weight/hr.

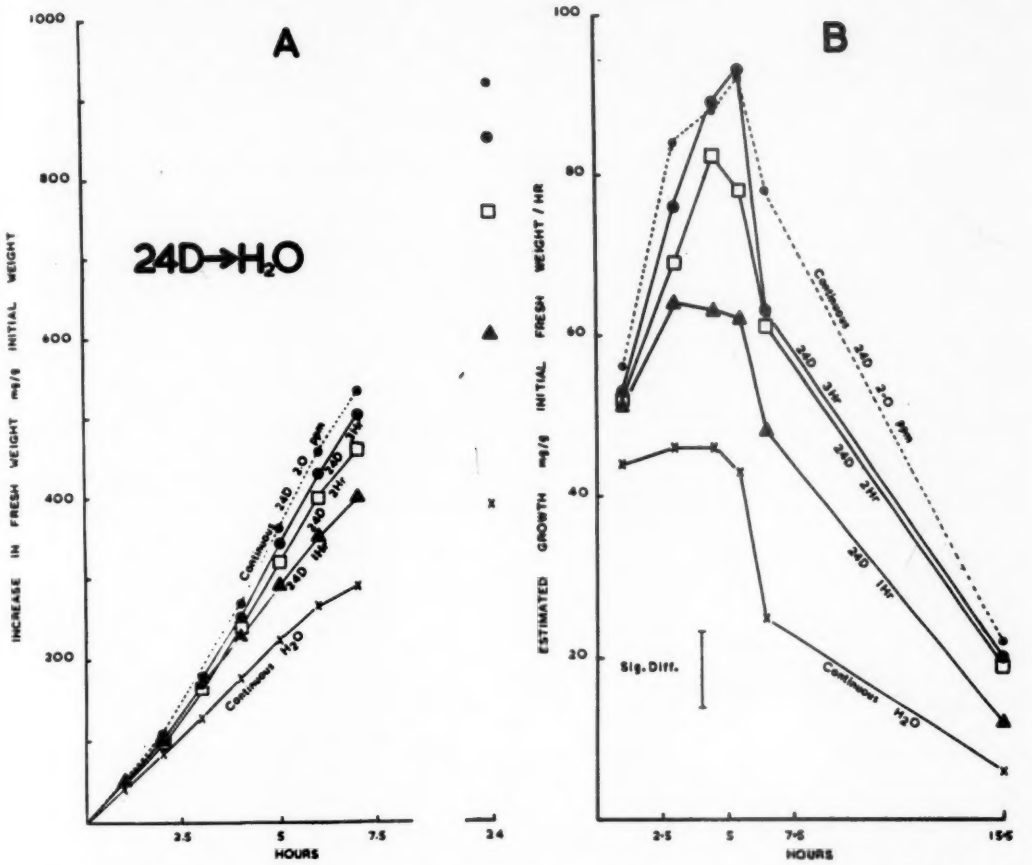

FIG. 3. A. Extension growth of sections of etiolated pea internode treated with 2,4-D 2.0 ppm for 1, 2 or 3 hours then transferred to H_2O, expressed as increase in fresh weight mg/g initial fresh weight.

B. The data from 3 A plotted as the estimated growth in mg/g initial fresh weight/hr.

then remains lower for the duration of the experiment. Sections kept continuously in IAA (12.5 ppm) maintained a higher growth rate than the control. Thus IAA, while first stimulating growth, may subsequently cause a depression in the growth rate. As the initial dose is increased, the fall in growth rate to a value below that of the control is delayed; when IAA is supplied continuously this response cannot be demonstrated (see also fig 1).

Many experiments were carried out over a wide concentration range with both 2,4-D (0.1 to 7.5 ppm) and 2,5-DBA (2.5 to 100 ppm) and in no instance was a depression of growth apparent after transference to water. Two experiments of this kind are shown in figures 3 and 4. These sections were treated with 2,4-D 2.0 ppm for 1, 2, 3 and 24 hours or with 2,5-DBA from 10 to 100 ppm for two hours and then transferred to water. Their rate of growth in water is seen to be either higher than or approaching that of control sections.

It is apparent, therefore, that the growth response of sections following a short treatment with IAA is different from that following a short treatment with either 2,4-D or 2,5-DBA.

It might be concluded that if a tissue is initially induced to grow more rapidly than the water control, it must, once the growth stimulus is removed, show a growth rate below that of the control since available substrates would have become depleted by virtue of the greater growth achieved. The fall in growth rate of IAA treated sections could be explained if there were a rapid depletion of IAA within the cells once the tissue is transferred to water. The response of 2,4-D and 2,5-DBA treated sections in which the growth rate remains either above, or falls to that of the control, could be the result of a continual growth stimulation by residual 2,4-D or 2,5-DBA. However, in a large number of experiments carried out over a wide range of suboptimal concentrations and times of exposure, these latter compounds have been found not

to induce growth rates below those of the controls, and where the rates do fall to that of controls, they may remain at that level for many hours. This suggests that the supply of substrates for further extension growth is not seriously impaired by small initial growth stimulations. This supposition is investigated further in the following experiments.

Response of Sections Initially Treated with IAA, 2,4-D or 2,5-DBA to Further Doses of IAA or 2,4-D: Weighed sections were treated with several concentrations of IAA, 2,4-D, 2,5-DBA and water for 1.75 hours in order to stimulate different amounts of growth. They were then transferred to distilled water for 3.5 hours, during which period the water was changed twice. At the end of this time each batch of sections was weighed and transferred to IAA 0.05 ppm. Twenty-four hours from the start of the experiment the sections were weighed again to obtain the total increase in fresh weight. The results for an experiment of this type, in which the increase in fresh weight of the tissue is expressed in mg/g of the initial weight of the sections is shown in table I.

For clarity, the initial treatment in water, IAA, 2,4-D or 2,5-DBA is henceforth referred to as a pretreatment.

Reference to table I shows that after 5.25 hours, the growth induced by the different concentrations of IAA, 2,4-D and 2,5-DBA covers a closely similar range of stimulations. When these sections are all transferred to the same concentration of IAA it is seen that the subsequent growth made by the sections pretreated with IAA is less than that made by those pretreated with water. The higher the initial dose of IAA (and hence the greater the amount of growth achieved by 5.25 hours) the smaller is the amount of growth in the subsequent IAA treatment. Sections pretreated with 2,4-D or 2,5-DBA however increased in fresh weight in the IAA posttreatments by an amount which approximates closely to that of the water pretreated sections, despite the fact that the pretreatments with these two compounds had caused increases in growth of the same order as those induced by the IAA pretreatment. This suggests that the greater amount of growth achieved in the first 5.25 hours by

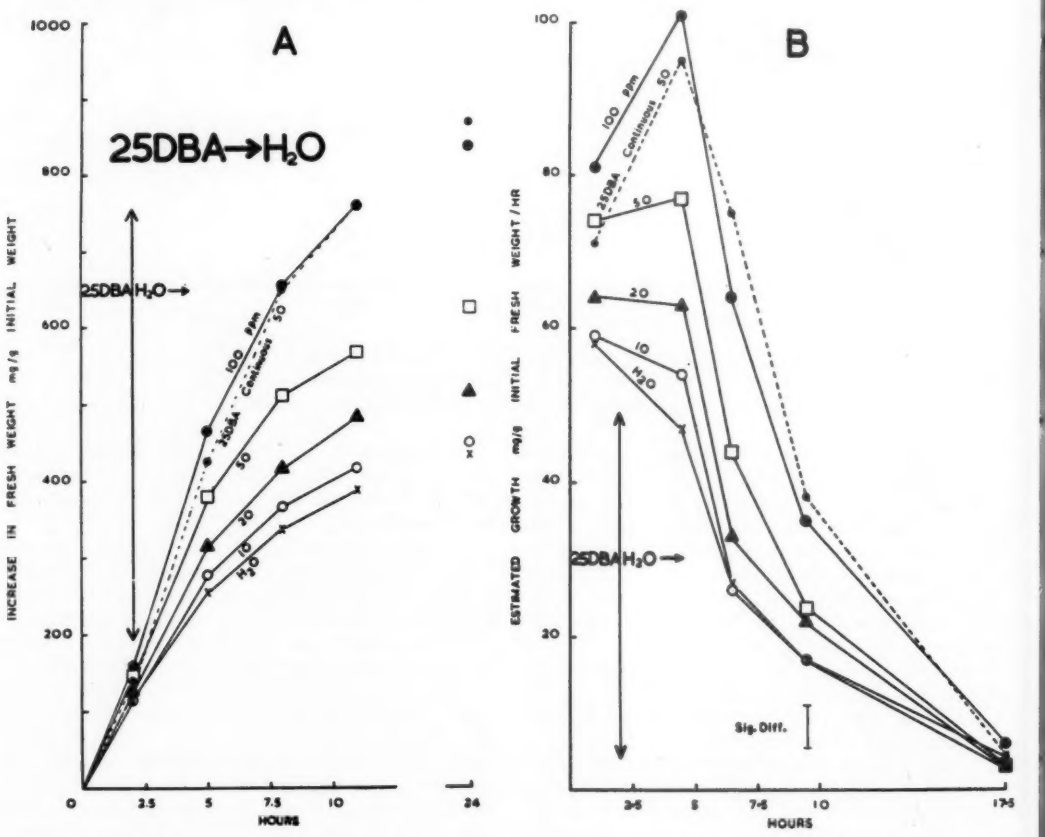

Fig. 4. A. Extension growth of sections of etiolated pea internode treated for 2 hours with different concentrations of 2,5-DBA followed by 22 hours in H_2O expressed as increase in fresh weight mg/g initial fresh weight.

B. The data from 4 A plotted as the estimated growth in mg/g initial fresh weight/hr.

TABLE I

INCREASES IN FRESH WEIGHT OF SECTIONS OF ETIOLATED PEA INTERNODE PRETREATED WITH VARIOUS CONCENTRATIONS OF IAA, 2,4-D, 2,5-DBA OR H_2O AND SUBSEQUENTLY TRANSFERRED TO IAA 0.05 PPM

PRETREATMENT		AVERAGE GAIN IN FRESH WEIGHT AS MG/G INITIAL FRESH WEIGHT			
		BY END OF PRETREATMENT	BY END OF 3.5 HR WATER WASH	BY END OF 18.75 HR IN IAA 0.05 PPM	DURING THE IAA POSTTREATMENT ONLY
		0–1.75 hr	*0–5.25 hr*	*0–24 hr*	*5.25–24 hr*
IAA	2.0 ppm	166	361	684	323
	1.0	152	335	723	388
	0.5	150	327	729	402
	0.25	134	298	735	437
	Mean	*151*	*330*	*718*	*388*
2,4-D	7.5 ppm	109	386	879	493
	3.0	104	343	836	493
	1.0	93	278	789	511
	Mean	*102*	*336*	*835*	*499*
2,5-DBA	100 ppm	114	363	904	541
	50	109	309	809	500
	20	96	250	739	489
	Mean	*106*	*307*	*817*	*510*
H_2O		85	254	732	489
Sig. diff. (P = 0.05) Between two treatments		17.1	33.0	49.2	55.2
H_2O vs 2,5-DBA mean or 2,4-D mean		14.0	26.9	40.2	45.1
H_2O vs IAA mean		13.5	26.1	38.9	43.6

those sections pretreated with 2,4-D or 2,5-DBA compared with those pretreated with water leaves the capacity for further growth unimpaired, whereas a pretreatment with IAA, which causes growth stimulations of the same orders of magnitude, in some way reduces the capacity of the tissue for further growth. It might be advanced that in the case of sections pretreated with 2,4-D and 2,5-DBA the growth made during the IAA posttreatment could be attributed to an additive stimulation given by IAA and by residual 2,4-D or 2,5-DBA within the tissue, and that, in the case of the IAA pretreatments, by the end of the water wash much, or all, of the IAA initially taken up by the sections would have been either lost again to the outside washing water or have been destroyed within the tissue. There would then be little or no additive stimulation during the posttreatment and a reduction in the growth capacity of the IAA pretreated sections compared with those pretreated with 2,4-D or 2,5-DBA should then be anticipated. The data might support this explanation for the growth response following IAA pretreatments, but one might also expect that if the growth response of the 2,4-D or 2,5-DBA pretreated sections are in part dependent upon residual quantities of these substances within the tissues, then the various concentrations of 2,4-D or 2,5-DBA used in the pretreatment should differentially effect the amount of growth during the posttreatment. This, in fact, is not the case. The amount of growth during the posttreatments would appear to be independent of the pretreatment concentration.

There is certain evidence that the pretreatment of plant tissue with growth regulators may effect the subsequent ability of the tissues to take up further growth substance from solution. Unpublished work by W. R. Birch in this Department has shown that a pretreatment of whole *Lemna minor* plants with 2,4-D caused changes in the subsequent uptake of labelled 2,4-D, while Reinhold (11) presents evidence that pretreatment with a high concentration (108.5×10^{-4} M) of 2,4-D depresses the metabolic, though probably not the physical, uptake of IAA by carrot discs. Such effects of pretreatments with growth regulators might be offered as an explanation for some of the results described in the present paper.

Thus, although there appears to be no effect of a 2,4-D pretreatment upon the subsequent total growth of sections transferred to IAA compared with that of sections pretreated with water (table I) the suggestion might be put forward that the smaller growth that occurs during the posttreatment in sections pretreated with IAA could be accounted for by a reduction in the uptake of growth substance during the posttreatment period. If this were so, then the growth during the posttreatment period of sections pretreated with a single concentration of IAA and then subjected to a range of posttreatment concentrations of IAA or 2,4-D should be proportionally lower than that of those pretreated with water and

TABLE II

AVERAGE FRESH WEIGHT INCREMENTS OF SECTIONS OF ETIOLATED PEA INTERNODE GROWING IN A RANGE OF CONCENTRATIONS OF IAA OR 2,4-D FOLLOWING A PRETREATMENT IN IAA OR H_2O

PRETREATMENT 2 HR	AV WT MG/10 SECTIONS AFTER H_2O WASH FOR 3.5 HR	POSTTREATMENT PPM	AV WT INCREMENTS MG/10 SECTIONS DURING POSTTREATMENTS	
			0–2.5 hr	*2.5–16 hr*
		IAA 0.25	46.5	71.5
		0.05	(A) 38.0	(A) 65.0
IAA 1.0 ppm	347.0	0.01	24.0	43.5
		H_2O	16.0	20.5
H_2O	305.5	IAA 0.25	65.0	87.0
		0.05	(B) 61.0	(B) 79.5
		0.01	45.5	55.0
		H_2O	34.5	34.0
		Weight increment differences (B – A)	*18.5*	*15.5*
			23.0	*14.5*
			21.5	*11.5*
			18.5	*13.5*
			Not sig. diff.	Not sig. diff.
			0–18.5 hr	
		2,4-D 7.5	112.0	
		2.5	(A) 106.5	
IAA 1.0 ppm	316.0	0.5	96.5	
		H_2O	17.0	
H_2O	276.0	2,4-D 7.5	135.0	
		2.5	(B) 132.5	
		0.5	120.5	
		H_2O	36.5	
			23.0	
		Weight increment differences (B – A)	*26.0*	
			24.0	
			19.5	
			Sig. diff. 4.86 (P = 0.05)	

given the same posttreatments. The results of an experiment given in table II show, however, that the difference in growth between the water and the IAA pretreated sections during their comparable posttreatments approximates to the same value and appears to be independent of whether the posttreatment is in water (in which case there can be no uptake of growth substance) or in any of the concentrations of IAA or 2,4-D.

The results of direct measurements of uptake during the posttreatments are presented in table III. Sections were pretreated for two hours with IAA, 2,4-D or water, placed in water for 3.5 hours and finally transferred to two concentrations of C^{14}-labelled 2,4-D for the posttreatment. After two and four hours in the labelled 2,4-D the sections were removed, weighed, and the total uptake of 2,4-D determined as radioactive C^{14}. The sections were combusted, the CO_2 collected in barium hydroxide and the barium carbonate counted under standard conditions. The author is indebted to Mr. E. Abeyaratne for carrying out all the analyses. The results demonstrate that there are no major effects of pretreatment with either IAA or 2,4-D on the 2,4-D uptake during the 1st four hours of posttreatment. Such data do not therefore support a view that the depression in growth which follows a short period of treatment with IAA can be explained by differences in the subsequent uptake of a growth regulator.

Further data for growth changes that take place during the course of an experiment are given in table IV. This lists the increments in fresh weight in mg/g initial fresh weight for a complete 24-hour period. The sections were pretreated with various concentrations of IAA, 2,4-D or with water and later transferred to a single concentration of IAA. By the end of the water wash growth of the IAA pretreated sections had slowed down and was less than that of the water pretreated sections. The increases in fresh weight induced by the various concentrations of IAA and 2,4-D were then of the same order of magnitude. The sections were then transferred to IAA 0.05 ppm. The growth increments for consecutive time periods following this transfer show that initially the IAA pretreated sections grow less than the sections pretreated with 2,4-D or water; sections pretreated with the highest concentration of IAA show the smallest growth increments. Another experiment is shown graphically in figure 5. Sections were pretreated with a single concentration of IAA, or 2,4-D or water for two hours. After the water wash batches of sections from each pretreatment were transferred to either

TABLE III

POSTTREATMENT UPTAKE OF LABELLED 2,4-D BY SECTIONS OF ETIOLATED PEA INTERNODE FOLLOWING PRETREATMENTS WITH IAA, 2,4-D OR H_2O

PRETREATMENT	AV FRESH WT MG/10 SECTIONS AFTER 2 HR PRETREATMENT FOLLOWED BY 3.5 HR H_2O WASH (AV INITIAL WT = 237.0)	POSTTREATMENT UPTAKE OF C^{14}-LABELLED 2,4-D EXPRESSED AS 10^{-7} MG 2,4-D/MG FRESH WT AT START OF POSTTREATMENT				
		2,4-D 1.0 PPM		2,4-D 3.0 PPM		
		0–2 hr	*0–4 hr*	*0–2 hr*	*0–4 hr*	*Uptake means*
IAA 1.0 ppm	336.5	6.53	10.98	19.00	31.63	*17.04*
2,4-D 1.0 ppm	315.0	7.26	11.07	17.26	32.68	*17.07*
H_2O	298.5	5.73	11.91	16.84	29.84	*16.08*
	Uptake means	*6.51*	*11.32*	*17.70*	*31.38*	

Effect of pretreatment during posttreatment—not significant at P = 0.01.
Effects of concentration and duration of posttreatment—significant at P = 0.01.

IAA 0.05 ppm or 2,4-D 2.0 ppm or to fresh distilled water. The average fresh weight increase in mg/batch of 10 sections is plotted for each consecutive time interval. Again it is seen that towards the end of the water wash the growth increments of the IAA pretreated sections are lower than those of sections pretreated in water. During the different posttreatments the IAA pretreated sections initially grow less than those pretreated with 2,4-D or water although the difference becomes less, the longer the sections remain in the posttreatment solutions.

DISCUSSION

A number of explanations may be proposed to account for the difference in the growth response of sections of pea internode following a short treatment with suboptimal concentrations of IAA, 2,4-D or 2,5-DBA and to account for the reduction in growth that occurs following the short treatments with IAA.

1. An effect on the IAA-oxidase system:

Galston and Dalberg (6) showed that the activity of the IAA-oxidase complex in brei preparations from etiolated pea stems is markedly increased when segments of the tissue are exposed to small doses of IAA or 2,4-D (10^{-7} M), but is decreased when higher concentrations are employed.

The doses of IAA and 2,4-D which have been used for the pretreatments in the present work are those which Galston and Dalberg found would partially inhibit the activity of IAA–oxidase; if this system were operative in controlling growth in the present experiments, one would expect the growth rate of IAA and 2,4-D pretreated sections to fall to a value

TABLE IV

SUCCESSIVE FRESH WEIGHT INCREMENTS OVER 24 HOURS OF SECTIONS OF ETIOLATED PEA INTERNODE PRETREATED WITH IAA, 2,4-D OR H_2O AND SUBSEQUENTLY GROWN IN IAA 0.05 PPM

PRETREATMENT	CONSECUTIVE FRESH WEIGHT INCREMENTS MG/G INITIAL FRESH WEIGHT SUCCESSIVE TIME INTERVALS IN HOURS										GAIN IN WEIGHT MG/G INITIAL FRESH WEIGHT		
	DURING PRETREATMENT	DURING H_2O WASH			DURING POSTTREATMENT IN IAA 0.05 PPM						0–5.5 HR	0–24 HR	DURING IAA POSTTREATMENT ONLY
	2	2	¾	¾	¾	¾	¾	¾	½	15			
IAA 0.5 ppm	176	142	18	18	54	54	44	56	36	258	358	860	502
1.0 "	184	162	17	21	41	35	36	47	27	237	384	807	423
2.0 "	207	167	14	14	36	36	32	44	32	227	402	809	407
Mean	*189*	*158*	*16*	*18*	*44*	*42*	*37*	*49*	*32*	*241*	*381*	*825*	*444*
2,4-D 2.0 ppm	124	158	41	59	77	69	59	55	33	263	382	938	556
3.5 "	126	181	48	53	72	64	52	54	29	257	408	936	528
5.0 "	134	190	55	59	71	59	59	49	29	278	438	983	545
Mean	*128*	*176*	*48*	*57*	*73*	*64*	*57*	*53*	*30*	*266*	*407*	*952*	*543*
H_2O	96	110	36	38	74	66	65	61	34	249	280	829	549
Sig. diff. (P = 0.05) Between two treatments	18.9	15.8	11.5	8.9	10.7	11.6	8.6	7.1	5.4	29.2	40.6	59.7	38.0
Between H_2O treatment and means	15.4	12.9	9.3	7.2	8.7	9.4	7.0	5.7	4.4	23.8	33.1	48.7	31.0

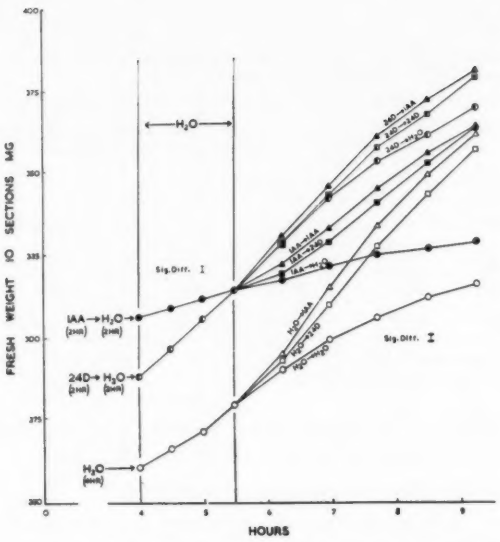

FIG. 5. Extension growth in mg/batch of 10 sections of etiolated pea internode treated for 2 hours with either IAA 1.0 ppm, 2,4-D 2.5 ppm or H_2O, followed by 3.5 hours in H_2O, then by a continuous posttreatment in either IAA 0.075 ppm, 2,4-D 2.0 ppm or H_2O.

which is higher than that of untreated controls when they are all transferred to water, since less IAA should be destroyed within the tissues. This is not so for IAA, since doses of 1.0 to 25 ppm for one hour cause a subsequent and increasing reduction in the growth rate to a value ultimately below that of the control. The continued higher rate which follows the more concentrated 2,4-D or 2,5-DBA pretreatments could be explained by residual growth substances in the tissues. However, the growth rates resulting from one-hour treatments with 2,4-D from 0.1 to 0.5 ppm (which should increase the oxidase activity) or 2,5-DBA from 2.5 to 10 ppm have been found, after a small stimulation for 4 to 5 hours, to fall to, and be maintained for many hours at, values which are not significantly different from the growth rate of the water controls. This suggests that if changes have occurred in the IAA–oxidase activity of these pretreated sections, the resulting effect on their further rate of growth does not fulfill the expectations of an adaptive enzyme system, even though the concentrations of IAA and 2,4-D employed are those with which Galston and Dalberg obtained marked effects on their brei estimations of IAA–oxidase. It would seem, therefore, that any changes that may occur in the oxidase activity of brei preparations of etiolated pea stems as a result of IAA or 2,4-D treatment, are not necessarily reflected in the subsequent extension growth or water uptake of the tissues in vivo.

2. Competition for essential growth metabolites:

The possibility that the growth capacity is reduced in sections which have been made to grow rapidly for the first few hours of an experiment has been discussed already and it is clear that the actual amount of growth achieved in these early stages does not necessarily reduce the growth capacity, since sections pretreated with 2,4-D or 2,5-DBA are still able to respond to further growth substance as readily as if they had grown only as much as water pretreated controls (table I). The reduction in growth that occurs following an IAA pretreatment must therefore be attributed to some special function of IAA. If some metabolic processes were stimulated more by IAA than by 2,4-D or 2,5-DBA, the competition for substrates between the various reactions could result in less of some essential factor being available to the elongation growth processes. This difference in the metabolic balance of the tissues might be a possible explanation for the reductions in growth that occur following an IAA pretreatment compared with pretreatments in 2,4-D, 2,5-DBA or water.

The results could, however, also be attributed to a production of growth inhibitory substances during the IAA treatment and this possibility is now explored more fully.

3. The formation of a growth inhibitor:

Further experiments have shown that when sections treated with IAA, 2,4-D or 2,5-DBA are washed and transferred at once to small volumes of water, biologically determinable amounts of growth stimulating substances pass out of the sections into the water. Since the growth rate may remain higher than that of water treated sections for many hours even when the sections are transferred to large volumes of water (fig 2 B, 3 B and 4 B), it is suggested that some of the originally applied growth regulator remains within the tissues and acts as a supply pool. Blackman (5) has found that *Lemna minor* plants treated with isotopically labelled 2,4-D lose 90 % of the 2,4-D initially taken up when they are transferred to a culture solution for three hours. Johnson and Bonner (7) also using labelled 2,4-D have shown that a certain fraction of the 2,4-D taken up by Avena sections is lost again from the tissue within minutes of the sections being transferred to water and that a further fraction of the 2,4-D remaining could be released only when the sections were transferred to unlabelled 2,4-D. However, they do not quote results for the growth of sections following the treatments. The present experiments with 2,4-D and 2,5-DBA suggest that as long as the growth regulator is present in the supply pool, extension growth remains above that of sections grown continuously in water, but once the pool is exhausted, growth falls to that sustained by endogenous auxin only, and is not significantly different from that of the water controls. When sections are treated with IAA, however, it is proposed that a stimulation of certain metabolic reactions may take place which results in the production of a substance which can dislocate the processes involved in growth by elongation. The observed

growth which follows an IAA treatment would then be the result of a balance between the growth promoting action of IAA, which may be similar to that of 2,4-D and 2,5-DBA, and the growth depressing action resulting from the development of the inhibitor. When sufficient IAA (or other growth regulating substances) are continuously present in the external solution (10), the supply pool is being continually replenished so that the proposed growth inhibitor may never attain a concentration high enough to cause a measurable reduction in growth. When IAA treated sections are transferred to water, the pool should steadily become exhausted until the only source of growth regulator is the auxin formed endogenously. The growth of these sections should then be less than that of the water treated sections because of their higher content of the suggested inhibitor. At a certain critical initial dose of IAA the amount of inhibitor formed should completely counteract the growth stimulating effect of the endogenous auxin and any residual IAA and the extension growth of the tissue should then cease (12.5 ppm IAA, fig 2, approaches this condition). A diagrammatic representation of such a system is shown in figure 6.

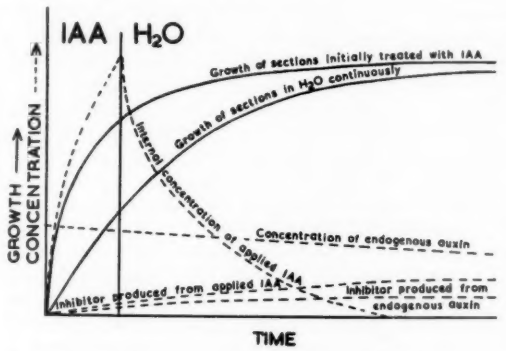

FIG. 6. Diagrammatic representation of the suggested growth stimulatory and growth inhibitory action of both endogenous auxin and applied IAA in sections of etiolated pea internode.

The difference in growth between IAA pretreated sections when they are given further IAA or 2,4-D and those pretreated with water has been shown to approximate to a constant (table II) and to be independent of the posttreatment concentration. This constant could reflect the amount of the growth inhibitor formed in the tissue as the result of the IAA pretreatment.

The development of a growth inhibitor in the pea sections treated with IAA offers a reasonable explanation for the results described. The evidence suggests that either the proposed growth inhibitor is not formed as the result of similar treatments with 2,4-D or 2,5-DBA or that if it is, the amount produced under the influence of these compounds is too small to be detected by the present methods. A survey of some of the data from other workers lends support to the proposed inhibitor hypothesis.

IAA in solution is readily decomposed by bacteria and it seems probable that in experiments involving very small volumes at low concentrations, the IAA may remain at the original concentration for a short while only. This, coupled with uptake by the test tissue, may cause the concentration to decline almost to zero, and might therefore offer conditions essentially similar to some of those described in the present paper. This might explain why sections of Avena coleoptile grown in very low concentrations of IAA (10^{-3} to 10^{-6} ppm) may show a growth stimulation when measured after five hours but a reduction in growth compared with a water control when measured after 20 hours (3).

It is also tempting to speculate that some of the effects ascribed to continuous low concentrations of IAA on the extension growth of roots may possibly be due to a loss of IAA from the external solution and the actual IAA treatment should, perhaps, be considered to be of short duration only.

While investigating endogenous rhythms in plants Ball and Dyke (2) measured the growth rate of whole seedlings of Avena which had previously been subjected to a short period of immersion in IAA and noted that the growth rate was initially stimulated but eventually fell below the level of control plants. Similar immersion treatments with 2,4-D resulted in growth stimulations but no subsequent growth inhibition, the rate falling only to that of the control values. They suggested that a growth inhibitor might be the cause of the reduced growth rate of the IAA treated plants, but did not extend their investigations further. Ball has, however, developed the view that IAA may induce the formation of a growth inhibitor in Aegopodium rhizomes, and has suggested that this might explain some of the effects of IAA on the geotropic responses of these organs (1). Bennet-Clark and Kefford (4) also suggested a conversion of IAA into an inhibitor to explain the rapid decrease in growth rate which succeeds the large initial growth stimulations in Avena sections which are exposed continuously to very high concentrations of IAA (70 and 100 ppm). They were, however, unable to demonstrate the diffusion of inhibiting substances into the external solution. It has been shown in the present work (fig 1 and 2 A) that growth inhibitions cannot be demonstrated while there is considerable IAA in the external solution, or when there is still enough IAA within the tissue to give appreciable growth stimulations. The presence of a growth inhibitor was therefore unlikely to be expressed in Bennet-Clark and Kefford's experiments as long as the sections were kept in high concentrations of IAA even if such a substance were to diffuse into the external solution. Thus it would seem that any attempt to demonstrate the diffusible nature of an inhibitor should be carried out when the IAA concentration within the tissue is minimal. Since the present work

has shown that growth inhibitions can be demonstrated after five hours, in pea sections treated for two hours with 1.0 ppm IAA, a number of experiments were carried out to determine if a diffusible inhibitor was present within such sections. Two principal methods were employed. The 1st method was carried out with the co-operation of Dr. C. C. McCready of this Unit, who developed the apparatus which was finally adopted.

One-cm pea internode sections were treated for two hours with 1.0 ppm IAA, washed and then grown for a further three hours either in water or on glass slides in a saturated atmosphere. A 2nd set of sections which received a water treatment were used as controls. At the end of the five hours the sections were weighed in batches of 10 and placed vertically through small holes in sheets of Perspex mounted over Petri dishes of water so that the greater length of the section was immersed in the water. Ten IAA and 10 water treated sections were mounted in this way in each dish. A disc of 2 % agar, 1 mm thick was then placed on the top (apical end) of each section and a 2nd Perspex rack with holes aligned above those in the lower rack was screwed into position. Freshly cut sections of pea internode were weighed and then dropped through these holes so that their basal ends were sealed to the upper surface of the agar disc below. The dishes were kept at 100 % humidity at 24° C and at the end of 24 hours the upper and lower sections were weighed again. The upper sections increased in weight by the same amount independently of whether the water they took up passed first through the IAA treated or through the water treated sections to which they were sealed. The lower water treated sections continued to take up water during the 24 hours, but the weight of the IAA treated sections remained practically unchanged. The inhibition of these latter sections was therefore effective, although no inhibition of growth occurred in the sections sealed above them indicating that no measurable diffusion of an inhibitor took place from the IAA treated sections.

A 2nd method was an attempt to collect the possible inhibitor by diffusion into a very small volume of water. Sections which had been treated for two hours with water or IAA at 1.0 ppm were washed, and then grown in water for a further three hours. Batches of 10 sections were arranged in the bottom of small beakers in the minimum amount of water (2.0 ml) and kept at 24° C and 5° C. At intervals from two to 24 hours, the sections were removed from a number of the beakers containing the water or IAA treated tissues. To the residual solution a freshly cut, weighed batch of 10 sections was added, and the increase in weight of these sections determined after 8 to 18 hours. No difference could be detected between the growth stimulating effects of the diffusates from the IAA and the water treated sections. It must be concluded, therefore, that if an inhibitor is formed within the tissue as a result of a treatment with IAA, it cannot be demonstrated by diffusion and it is possible that it may influence only the growth of the cells in which it is produced.

The Role of an Inhibitor in the Intact Plant: Evidence has been presented which supports the proposal that IAA may have a dual action in controlling growth when it is applied to sections of etiolated epicotyl. It remains to consider how this concept might help to explain the growth regulating mechanism of an intact plant.

It is generally accepted that there is a gradient of auxin from the shoot apex. As cells differentiate, they become displaced from the apex and may therefore receive a continually falling supply of auxin. During this period it is suggested that the growth inhibitor may slowly accumulate and finally reach a concentration which counteracts the auxin present. At this stage, extension growth of the cell should stop. It may be demonstrated, however, that such tissues still possess a capacity to grow, for if they are excised and placed in relatively high concentrations of IAA, further extension growth will occur (7).

The formation of a growth inhibitor following the treatment of a plant with IAA might offer an additional explanation for its poor herbicidal activity compared with other synthetic growth regulating substances, although ease of penetration, photochemical decomposition and bacterial breakdown should also be considered. The apparent failure of 2,4-D to induce the formation of an inhibitor together with its relative stability within the plant may explain why completely unrestrained growth results from an application at herbicidal concentrations.

It is therefore proposed that the formation of a growth inhibitor might be one of the ways in which the plant has developed an elegant safety mechanism to protect itself from its own production of auxin and that this safety mechanism is not developed against completely foreign synthetic growth substances such as 2,4-dichlorophenoxyacetic or 2,5-dichlorobenzoic acids.

Summary

1. A simple, rapid and reliable method is described for measuring small increments of growth in sections of etiolated pea internode. The results can be interpreted in terms either of extension growth or increase in fresh weight.

2. The growth of sections kept continuously in solutions of suboptimal concentrations of IAA, 2,4-D or 2,5-DBA for 24 hours, was, over measured periods of time, always either greater than, or the same as that of sections kept in water.

3. The growth of sections treated for a few hours only with suboptimal concentrations of IAA and then transferred to water for the remainder of the 24 hours, ultimately fell below that of the water controls. The reduction in growth that occurred and the time at which it became apparent were dependent upon the concentration of the original IAA treatment.

4. No reduction in growth was observed in sec-

tions similarly treated with 2,4-D or 2,5-DBA. Over measured intervals of time growth remained either higher than or equal to but was never less than that of the water controls.

5. Sections which received an initial treatment with IAA did not respond as much to further applications of a growth regulator as sections which had received an initial treatment with either 2,4-D, 2,5-DBA or water.

6. The nature of the growth depression which follows an IAA treatment is discussed. It is suggested that IAA may have a dual role in controlling growth. It may function as a growth promotor in a way similar to that of 2,4-D or 2,5-DBA. In addition, it may stimulate the formation of a growth inhibitor. It is proposed that one of the effects of IAA upon cell metabolism is the formation of a substance which accumulates within the tissue and which can in some way dislocate the growth processes and render the tissue less sensitive to further applications of growth regulators. The formation of such an inhibitor could be a safety mechanism to protect the plant from its own production of auxin, and might offer a partial explanation for the weak activity of IAA when applied to intact plants.

7. The results suggest that pea sections treated with 2,4-D or 2,5-DBA either do not develop this safety mechanism or develop it only to a small extent and it is proposed that this difference in response to IAA and the synthetic growth regulators might be a further factor to account for the much greater effectiveness of the latter compounds in whole plants.

The author wishes to thank Miss Pamela Clements and Mrs. Ruth Kasasian for technical assistance, and the Unit of Biometry, Oxford, for their advice on statistical analyses. She also thanks Professor G. E. Blackman for his suggestions and encouragement and Dr. E. K. Woodford and other members of the Agricultural Research Council Unit of Experimental Agronomy for many helpful discussions.

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THE RELATION OF THE COTYLEDONS TO ROOT DEVELOPMENT OF PINE EMBRYOS GROWN IN VITRO[1]

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Attempts to obtain successful seed germination of certain conifers have resulted in the adoption of modified cultural techniques. Aside from certain commonly practiced procedures, e.g., stratification, some workers have attempted, and have been successful in some instances, in growing excised embryos to fairly large trees. Embryos are initially cultured on artificial media with or without the surrounding nutritive female gametophytes, and then transplanted to soil as done by Stone and Duffield (16) and Haddock (5). If successful, this technique also has its obvious advantages for forest geneticists who may wish to obtain seedlings of hybrid origin from a small crop of seeds. The realization that embryos can be grown outside their normal environment has likewise presented

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morphologists with interesting working material for the experimental approach to problems of histogenesis and organogenesis (1, 2).

It is not the intent of the authors to review all of the literature on embryo-culture but rather to discuss only those works which are pertinent to the present investigation. Bulard (3) reported that normal growth of excised Ginkgo embryos was obtained only when the cotyledons were in contact with the nutrient media, thereby serving a haustorial function by supplying glucose to other parts of the young plant. Earlier Li (7) reported that Ginkgo embryos could be grown in vitro, with or without the cotyledons in a nutrient medium, although growth of the cotyledons, hypocotyl and primary root virtually ceased after 15 days. Bulard reported that growth of the primary root of Ginkgo took place even though the cotyledons were not in contact with the nutritive medium. The shoot, however, did not grow. More recently Sacher (13) described in detail the developmental pattern of mature sugar pine embryos (*Pinus lambertiana* Dougl.) in vitro, and in contrast to Ginkgo, clearly showed that the haustorial function of the cotyledons in Pinus was not essential for initial growth of the primary shoot. Of particular interest in Sacher's work, however, was the observed failure of continued root growth when the excised embryos were planted with their radicle ends submerged beneath the nutrient agar medium.

The present study confirms, in general, the observations of Sacher but also demonstrates the important role of the cotyledons in affecting root growth of pine embryos grown in vitro. Therefore, the primary purpose of this paper is to point out the beneficial influence of sucrose on root growth and development when supplied through the cotyledons, and to report briefly the effects of other factors such as IAA (indole-3-acetic acid), gibberellic acid, and TIBA (2,3,5-triiodobenzoic acid) on the development of mature pine embryos under aseptic cultural conditions.

Experimental Techniques

Mature seeds of *P. lambertiana* from El Dorado County, California, were stored dry for approximately 6 months at 5° C prior to their use. The seed coats were removed by hand without injury to the

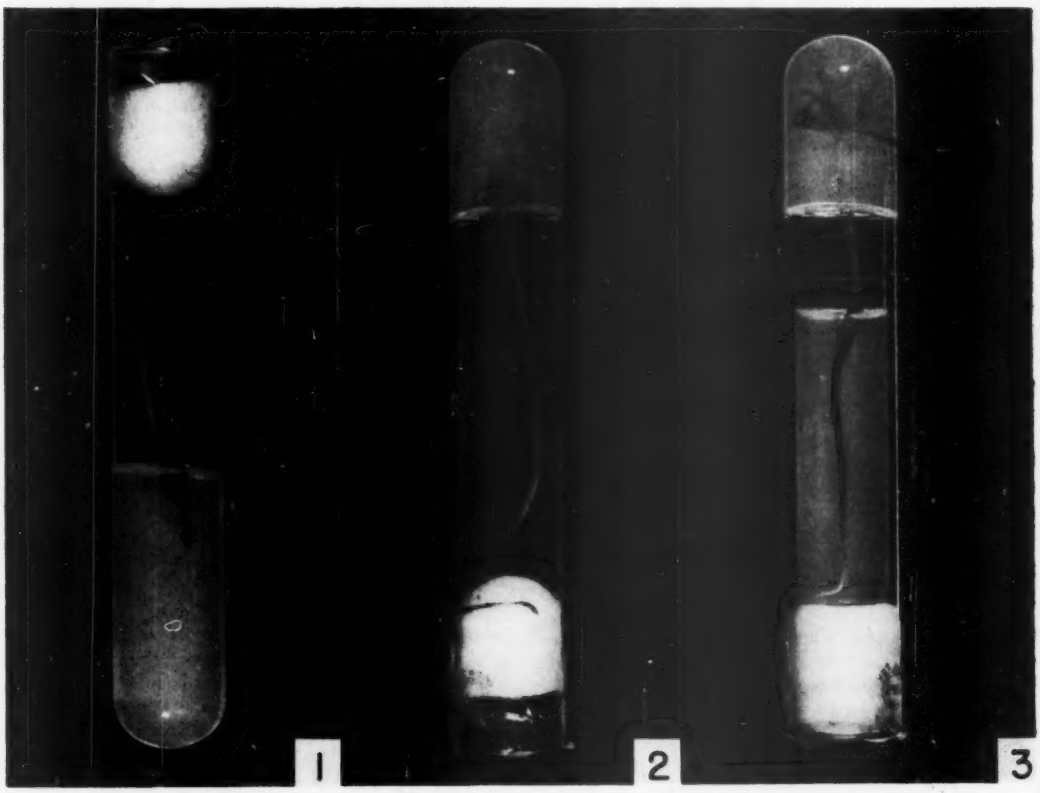

Fig. 1. Normally oriented embryo (*P. lambertiana*) on sucrose medium at 30 days. × ¾.

Fig. 2. Embryo with cotyledons embedded in sucrose medium, root in air. Note length of hypocotyl and root at end of 30 days. × ¾.

Fig. 3. Embryo 14 days old with cotyledons in sucrose medium and root growing in plain medium. × ¾.

megagametophyte and the latter surface sterilized in a 7 % solution of "Pittchlor" (active ingredient, calcium hypochlorite, obtained from the Columbia-Southern Chemical Corporation, Pittsburgh, Pennsylvania) for 5 to 10 minutes. After sterilization, the intact megagametophytes were transferred to a beaker of sterile double-distilled water for a period of three to four hours. During this time the gametophytes imbibed a considerable quantity of water which made the subsequent removal of embryos considerably less difficult. Upon aseptic removal, the excised embryos were immediately transferred to the sterile 1.0 % agar media.

The basic culture medium was prepared, using double-distilled water, Knop's solution as modified by Gautheret (4) and Nitsch (12), and the essential micronutrients according to Nitsch (12). Nutrient iron in the form of ferric citrate was supplied at a concentration of 10 mg/l. Two types of culture media were used: 1) the medium described above without any organic supplement, hereafter referred to as plain medium, and 2) the same medium containing 2 % sucrose as the only organic supplement, termed sucrose medium. In other experiments IAA, gibberellic acid, and TIBA were added to the sucrose media as previously indicated. After preparation of the different media the acidity was adjusted to a pH of 5.5 and approximately 15 ml of the agar medium allotted to each 25 × 150 mm culture tube. These were plugged with non-absorbent cotton, and autoclaved under 15 pounds pressure at 110° C for 15 minutes.

The excised mature embryos were planted in the agar media in two ways 1) with the cotyledons beneath the surface of the agar leaving the hypocotyl and radicle end free, or 2) with the radicle end and basal part of the hypocotyl submerged in agar leaving the upper part of the hypocotyl and cotyledons free. In either case the culture tubes were oriented so that the radicle was always directed downward; this was accomplished by merely inverting the tubes of the 1st type. To prevent excessive water loss, the test tubes were capped with a thin layer of polyethylene plastic film in addition to the cotton plugs. The cultures were placed in a standard tissue-culture laboratory in which both incandescent and fluorescent light was supplied under a 12-hour photoperiodic regime. Temperature was maintained at 24° ± 1° C during each successive experiment. Under the methods described it was possible to explant and grow successfully numerous embryos with little contamination from fungi or bacteria. In no experiment did the losses exceed 10 %; normally they were considerably less.

Discussion of Results

Earlier studies (unpublished work of Claud L. Brown) in which the mature embryos of longleaf pine (*P. palustris* Mill.) were cultured successfully on a sucrose medium by submerging the radicle of the embryo into the medium resulted in the same type of root development as that described by Sacher (13). That is, initial root growth was rapid during the first two to five days in culture, after which growth of the root became noticeably retarded, reaching lengths of only 1 to 2 cm by the end of two to three weeks. Further root growth was negligible and the roots characteristically turned dark brown by the end of the 4th week. Identical experiments were performed in the current study with *P. lambertiana* embryos and essentially the same results were obtained, confirming the observations of Sacher (fig 1).

It was found in subsequent experiments, however, that embryos planted with their cotyledons in the sucrose medium exhibited more rapid rates of initial root growth than those planted in a normal manner,

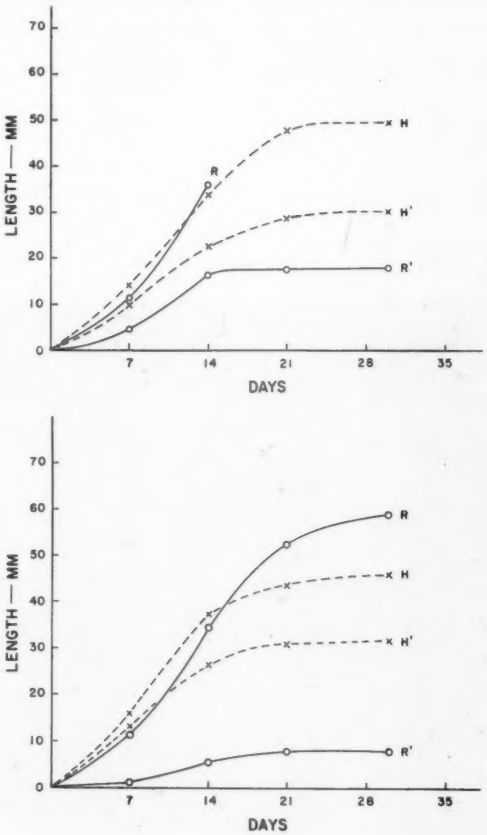

Fig. 4 (*top*). Course of growth of embryos during first 30 days on sucrose medium when oriented (1) with cotyledons in the medium (R, roots; H, hypocotyls) and (2) with radicle in medium (R′, roots; H′, hypocotyls). Each point on curves represents mean of 48 embryos.

Fig. 5 (*bottom*). Course of growth of roots and hypocotyls of 30-day-old embryos when (1) cotyledons were placed in sucrose medium and roots in plain medium (R, roots; H, hypocotyls) and, (2) cotyledons in plain medium with roots in sucrose medium (R′, roots; H′, hypocotyls). Each plotted point represents mean of 48 embryos.

i.e., with the radicle submerged in the same medium. Indeed, in the former, the roots continued to grow downward in air until they reached the cotton plug but finally died of desiccation by the end of three to four weeks (fig 2). Embryos planted in this manner also showed a significant increase in hypocotyl elongation, although there was little difference in the final length of the cotyledons. A comparison of the quantitative differences between the two methods of treatment is shown in figure 4.

Further evidence that sucrose entering the embryos through the cotyledons greatly promoted root elongation was obtained in the following manner. The embryos were initially placed with their cotyledons in the two types of agar media (plain and sucrose) and at the end of 7 days, after embryo growth had begun, another source of agar was applied to their basal or radicle ends. This was accomplished by inserting a vial of sterile agar inside the culture tube so that the root was completely submerged beneath the surface. Thus, each embryo could be grown aseptically with both the cotyledons and root surrounded by an agar medium. In this manner, sucrose could be supplied to either end of the embryo while the opposite end was contained in the plain agar medium. This method of culture proved highly successful because the desiccation of roots was no longer a problem and the effect of sucrose per se on root growth in agar could now be specifically determined.

In several replicated tests it was found that each time sucrose was added to the medium in which the cotyledons were embedded, the primary root grew quite rapidly into the plain agar medium below. The initial rate of root growth was not, however, as high in the plain agar as it was previously in air; yet the final lengths attained were much greater in agar because growth continued considerably longer. In fact, some of the roots, upon reaching the bottom of the inner vials began to grow in a spiral manner as shown in figure 3. It was also of interest to note that even though the rate of root growth decreased with time, the 1st two or three weeks being the most rapid, there was no dark brown discoloration of the roots now growing beneath the agar surface. Even after a period of 6 to 8 weeks the root tips (2- to 3-mm portion) appeared normal in both color and form.

In striking contrast to these observations, root growth was practically nil when the cotyledons were placed in the plain agar medium, and the roots supplied with a source of sucrose as shown in figure 6.

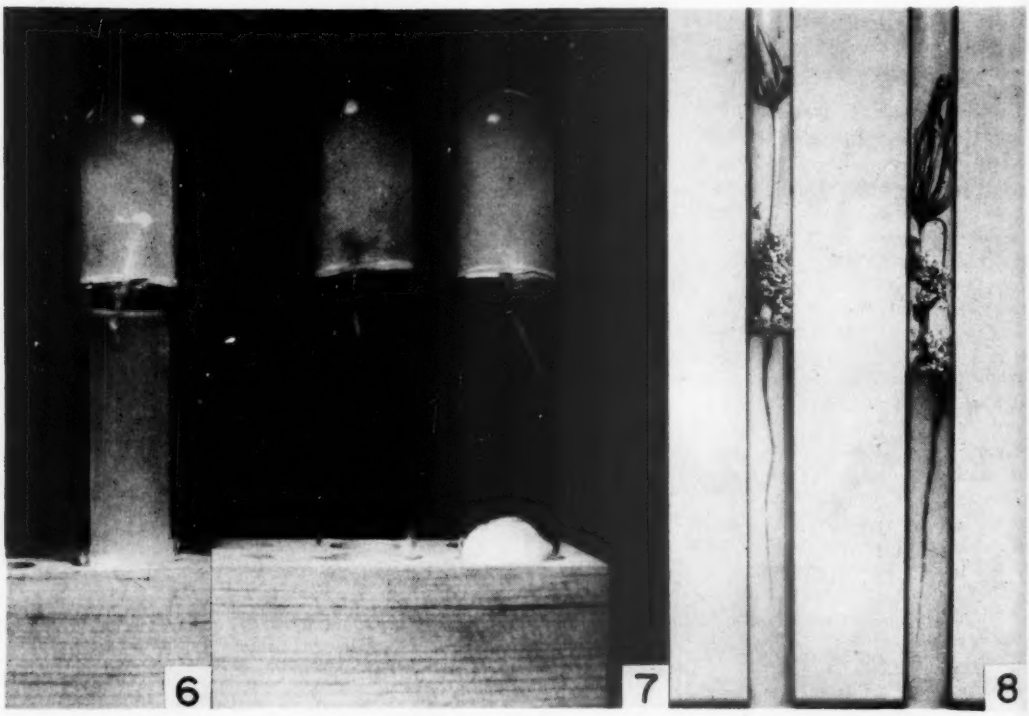

FIG. 6. Embryo, 14 days old, with cotyledons in plain medium and the root in sucrose medium. × ⅔.

FIG. 7. Embryos at the end of 14 days. Cotyledons in sucrose medium, left; cotyledons in plain medium right. Note hypocotyl elongation, without root growth in embryo on right. × ⅔.

FIG. 8. Embryos at 21 days on plain medium with megagametophyte still attached to tips of cotyledons of plant on right. × ⅓.

These differences in response, reflecting the manner in which sucrose is supplied to the embryos, are expressed in figure 5.

Other experiments in which the mature embryos were cultured for short periods (ca. 2 to 3 weeks) on plain medium alone, proved of considerable interest with respect to factors affecting the initial growth response. For example, when the cotyledons were placed beneath the surface of the plain agar medium, leaving the root free in air, subsequent hypocotyl elongation was quite rapid reaching lengths of 20 to 30 mm during the 1st three weeks, whereas in every case the root failed to grow at all (fig 7). Conversely, when the embryos were planted with the radicle end submerged in the plain medium, initial root growth did occur, and almost to the same extent as it did on a sucrose medium (fig 1), while hypocotyl elongation was somewhat reduced. These observations suggest that excised mature embryos of *P. lambertiana* contain enough food reserves to make an initial growth response, and that this growth may be transferred, within limits, from one organ to another apparently depending upon which portion of the embryo first receives available moisture for active cellular metabolism. Obviously, after this initial food reserve is exhausted a source of carbohydrate is essential for survival of embryos planted in either way, and as previously demonstrated the manner in which it is supplied primarily determines the extent of further root development.

These studies have shown that external nutritional conditions alone are not the primary factors limiting root growth of pine embryos in vitro; neither can the failure of normal root growth in nutrient agar be attributed wholly to such factors as low oxygen tension, pH changes, osmotic conditions, or the accumulation of toxic substances. Rather it seems quite clear that a carbohydrate, in this case sucrose, when made available to the cotyledons is effectively metabolized so that either 1) elaborated substances essential for continued root growth are transported basipetally through the hypocotyl to the root or 2) some type of inhibition is removed. The megagametophyte of the young germinating seed in vivo accomplishes the same results by supplying an abundant source of carbohydrate to the cotyledons. This was demonstrated in the present study by obtaining excellent root growth in long tubes of agar (fig 8) when the intact megagametophyte was placed in sterile vermiculite just above the plain agar in a manner somewhat similar to that previously described by Stone and Duffield (16).

It should be noted that Sacher (13), using a different technique than the one reported here, observed abnormal development of mature embryos of *P. lambertiana* when they were submerged beneath the surface of the agar medium, inasmuch as they commonly formed a tumorous type of growth or did not grow at all. From this it was suggested that the apparent differences in response between the embryos of Ginkgo and Pinus when cultured beneath or on an agar surface, were probably other than nutritional and could perhaps be attributed to differences in respiratory rates of the two species. The authors, however, do not draw the same inferences since it is apparent that the embryos of *P. lambertiana* can be grown successfully in a manner comparable to that of Ginkgo as described by Bulard (3). In fact, the resultant growth rates more closely approached those of the in vivo cultures.

Effect of Other Factors on Root Growth

Indole-3-Acetic Acid: The inhibition of root growth by low concentrations of auxin is a well known phenomenon (18). Slankis (15) observed that concentrations of naphthaleneacetic or indole-3-acetic acid above 0.5 mg/l commonly inhibited the growth of excised long roots of pine in liquid culture, while concentrations below 0.05 mg/l promoted elongation. Sacher (13) also demonstrated the inhibitory effects of naphthaleneacetic acid (0.5 mg/l) on root growth of pine embryos in nutrient agar. This concentration caused the formation of protuberances on the primary roots, which Sacher interpreted as short roots.

In the current studies it seemed appropriate to test the effect of IAA in plain agar when applied directly to the primary root in concentrations of 0.1, 1.0, and 10.0 mg/l, while the cotyledons were submerged in the nutrient sucrose medium. At the end of 14 days root inhibition was not significant at 0.1 mg/l while it was very pronounced at 1.0 and 10.0 mg/l (table I). The inhibition could also be demonstrated by supplying IAA through the cotyledons with the sucrose at the same concentrations. None of the primary roots, however, developed apparent short roots even after 60 days in culture, although, the tips of those receiving 10.0 mg/l became swollen by the end of 7 days and thereafter, at this concentration, root growth was greatly retarded. The roots receiving 1.0 mg/l were somewhat contorted but produced no protuberances resembling short roots, while those growing in agar with 0.1 mg/l appeared normal in every respect (fig 9). In comparing these results with those of Sacher (13) it appears that sucrose supplied through the cotyledons does have a modifying effect on the degree of inhibition exerted by IAA, particularly in the expression of morphological characteristics such as protuberances or short roots.

Table I

Inhibition of Root Growth of 14-day-old Embryos Grown with Cotyledons in Sucrose Medium and Roots in Plain Medium Containing Different Concentrations of IAA *

Controls	IAA 0.1 mg/l	IAA 1.0 mg/l	IAA 10.0 mg/l
mm	*mm*	*mm*	*mm*
33.5 ± 2.7 **	29.7 ± 2.1	23.8 ± 1.7	13.3 ± 1.3

* Each value is mean of 48 individual embryos.
** Standard error.

GIBBERELLIC ACID: The pronounced effect of the gibberellins on stem and leaf elongation in many species of higher plants has been demonstrated by numerous workers. (See review by Stowe and Yamaki (17).) Only a few studies, however, have been specifically concerned with the response of coniferous species to applications of these biologically active compounds. Marth, Audia, and Mitchell (8) were among the first to observe the effect of gibberellic acid on several conifers and to report its general ineffectiveness on the growth of the species tested, namely, *Pinus taeda* L., *P. virginiana* Mill., *P. strobus* L., *Picea glauca* (Moench.) Voss., and *Juniperus chinensis* f. *pfitzeriana* (Spaeth) Rehder. More recently, Nelson (9) reported that a 1.0% solution of gibberellic acid applied in lanolin paste to the active apices of eastern white pine (*P. strobus* L.) caused a small but non-significant increase in height growth, while Arizona cypress (*Cupressus arizonica* Greene) failed to respond at all. In fact, the latter appeared slightly inhibited by the treatment. The work thus far would certainly indicate that the conifers, taken as a group, are much less responsive to applications of gibberellic acid than most other plants which have been tested.

Although some of the most striking effects of gibberellic acid are those associated with increased shoot extension, there has also been a considerable amount of work devoted to its effect on root growth (17). Most workers seem to agree that the gibberellins exert a detrimental effect on the underground portions, although as pointed out by Stowe and Yamaki (17), there are also some reports that root growth may be promoted. In a more recent study, Whaley and Kephart (19), working with excised roots of maize, observed a beneficial response in one inbred line at concentrations of 10, and 20 mg/l, whereas another inbred showed no response at the same concentrations. The hybrid of the two inbred lines gave essentially the same response as the 1st inbred line.

In the current studies, gibberellic acid was added to the sucrose media in concentrations of 0.1, 1.0, and 10 mg/l. It was supplied through the cotyledons in some cultures by the manner previously described, whereas in others it was applied directly to the roots in sucrose medium. After 30 days there was no significant increase in either cotyledon or hypocotyl elongation at any of the concentrations used, although there was an apparent stimulation of root growth (fig 10), at the two higher concentrations during the initial 14-day culture period (table II). After this period, the rate of root growth paralleled that of the controls, but there seemed to be less browning of the roots in the cultures containing gibberellic acid. Even after three to four months most of the root tips appeared active. Other aspects of embryo development did not differ from the controls; certainly the characteristic leaf and shoot responses obtained by gibberellic acid in some plants was not observed in the embryos of *P. lambertiana* grown in vitro. It should be pointed out in interpreting the results of this experiment that the gibberellic acid was autoclaved with

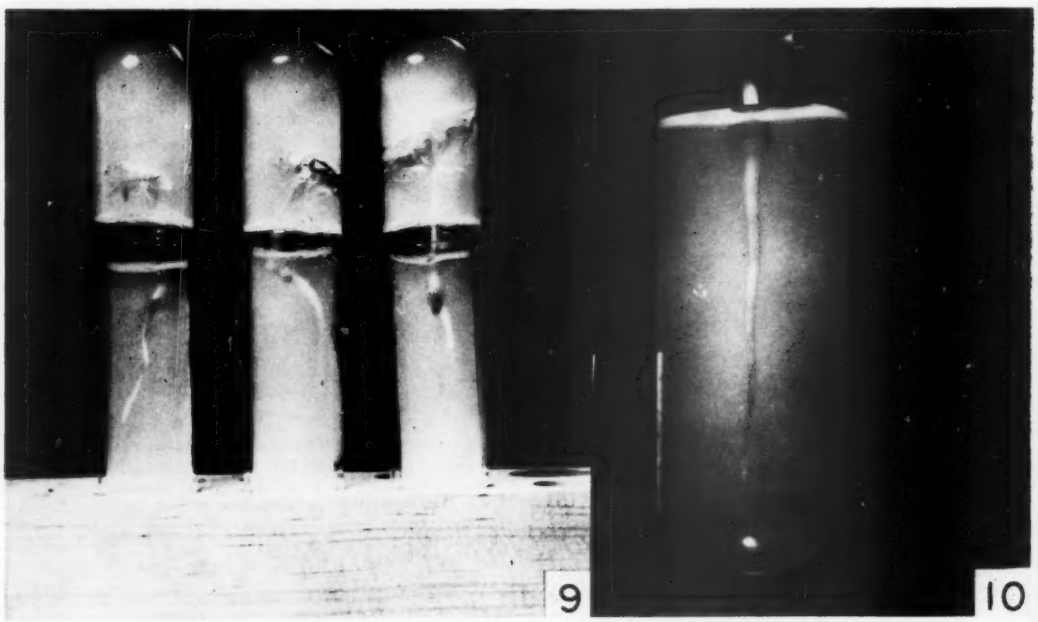

FIG. 9. Embryos at 14 days, showing the degree of root inhibition caused by the addition of IAA to the plain basal medium at concentrations of (a) 0.1 mg/l, (b) 1.0 mg/l, and (c) 10.0 mg/l, left to right respectively. ×⅔.

FIG. 10. Root growth in sucrose media supplemented with gibberellic acid (10.0 mg/l) at end of 30 days. ×1.

TABLE II

COMPARISON OF ROOT GROWTH OF EMBRYOS ON SUCROSE MEDIA CONTAINING DIFFERENT CONCENTRATIONS OF GIBBERELLIC ACID. (ROOTS SUBMERGED BENEATH THE MEDIA, COTYLEDONS IN AIR) *

DAYS	CONTROLS	GA 0.1 MG/L	GA 1.0 MG/L	GA 10.0 MG/L
	mm	*mm*	*mm*	*mm*
7	4.7 ± 1.2 **	5.8 ± 1.1	6.4 ± 1.7	6.4 ± 1.8
14	16.3 ± 1.9	17.5 ± 0.6	23.5 ± 2.5	23.7 ± 2.8
21	17.5 ± 2.1	18.0 ± 2.3	24.7 ± 3.2	25.1 ± 3.1
30	17.6 ± 2.5	18.2 ± 2.2	25.1 ± 3.5	25.3 ± 3.0

* Each value is mean of 48 individual embryos.
** Standard error.

the nutrient media for 15 minutes under 15 pounds pressure which may have considerably reduced its biological activity. Yet, Schroeder and Spector (14) using the same cultural techniques, i.e., autoclaved gibberellic acid, report a marked increase in weight of excised fruit tissue in vitro corresponding to an increase in concentration of gibberellic acid up to 25 mg/l in the presence of indole-3-acetic acid, and up to 5 mg/l in the absence of the latter. Above these concentrations gibberellic acid caused a pronounced inhibition of callus growth and a concomitant decrease in weight. Whether or not autoclaving reduces the activity of gibberellic acid to an appreciable extent or whether a possible conversion to another active substance(s) occurs in the nutrient media is not presently known.

TIBA: Reports of the effects of 2,3,5-triiodobenzoic acid (TIBA) on growth correlations in higher plants are numerous. Its role as a so called "anti-auxin" has recently been attributed, at least in part, to a direct effect on the polar auxin transport system by Niedergang and Skoog (10, 11) and Kuse (6). In the present studies, TIBA was added to the sucrose medium at the concentration of 10.0 mg/l and the embryos were planted in the medium in the manners previously described. Embryo growth was noticeably retarded in all cases, resulting in the diminution of both hypocotyl and root elongation (table III). After the embryos had been in culture for 30 days, auxin

TABLE III

EFFECT OF TIBA ON HYPOCOTYL AND ROOT GROWTH OF EMBRYOS PLANTED WITH COTYLEDONS IN SUCROSE MEDIUM AND ROOTS IN PLAIN MEDIUM *

DAYS	CONTROLS (SUCROSE)		SUCROSE PLUS TIBA 10.0 MG/L	
	hypocotyls	*roots*	*hypocotyls*	*roots*
	mm	*mm*	*mm*	*mm*
7	15.0 ± 1.2 **	7.0 ± 0.9	7.8 ± 1.0	0.0 ± 0
14	19.8 ± 1.6	35.0 ± 2.8	8.5 ± 1.1	2.0 ± 0.1
21	23.5 ± 2.1	40.0 ± 3.1	12.2 ± 1.3	9.5 ± 0.7
30	38.0 ± 3.2	49.1 ± 3.4	17.0 ± 1.8	15.0 ± 1.2

* Each value is mean of 48 individual embryos.
** Standard error.

transport tests were made on hypocotyl segments by placing donor blocks containing 2.0 mg/l IAA in 1.5 % agar on the apical cut end of segments 0.5 cm in length, and plain agar receiver blocks at the basal end for a four hour period. The basal blocks were assayed for auxin by the standard Avena curvature test as described by Went and Thimann (18), and no measurable transported auxin was obtained in any of the TIBA treated embryos. Untreated segments transported IAA in amounts yielding 8 to 12 degrees Avena curvature during the same period. The loss of polar auxin transport in the hypocotyls of pine embryos placed on a sucrose medium containing TIBA substantiates the conclusions reached by Niedergang and Skoog (11), and further indicates that basipetal polar transport is essential in affecting the normal growth response of intact embryos.

SUMMARY

1. Mature seeds of *P. lambertiana* Dougl. were placed in dry storage for approximately 6 months at 5° C prior to their use. Seed coats were removed and the megagametophytes surface-sterilized. Embryos were excised aseptically and oriented on agar media in two ways: 1) with the radicle in agar, and 2) with the cotyledons in agar, leaving the radicle free in air. Culture tubes of the latter series were kept inverted so that the root was always directed downward. The basic medium was a modified Knop's solution to which 2 % sucrose and other substances were added as specified.

2. Embryos grown with the radicle embedded in a sucrose medium exhibited immediate growth of all organs; the roots, however, grew only 1 to 2 cm in length during the first two to three weeks and thereafter became completely inhibited. In contrast, when the cotyledons were placed in the sucrose medium, leaving the radicle end free in air, growth of the root was rapid, reaching 5 to 8 cm in length before dying of desiccation. In additional experiments, root desiccation was overcome by inserting the roots of the latter type into a sterile vial of agar placed inside the original culture tube and excellent root growth was obtained for much longer periods. If, however, the two types of media were reversed, i.e., if the plain medium were supplied to the cotyledons and the sucrose medium applied to the radicle end or root, the latter grew only 1 to 2 cm in length and again became completely inhibited. Thus, it was clearly demonstrated that a carbohydrate, sucrose in these experiments, when supplied through the cotyledons exerted a much greater beneficial effect on both rate and duration of root growth than when it was supplied to the embryo directly via the root. Under in vivo conditions, the metabolite is, of course, supplied through the cotyledons by the megagametophyte.

3. The failure of root growth to occur beneath an agar medium when embryos were planted in a normal manner (i.e., radicle submerged beneath the agar) was not attributed to such factors as low oxygen tension, changes in pH, accumulation of toxic sub-

stances, etc., but rather to the manner in which the carbohydrate source was supplied to the excised embryo.

4. The effects of certain other substances were tested. With sucrose supplied through the cotyledons, the inhibitory effect of IAA on root growth was somewhat modified. There was an initial stimulation of root growth by the addition of gibberellic acid, but the effect was not sustained. The shoots did not exhibit a beneficial response. Embryo growth was retarded by TIBA (2,3,5-triiodobenzoic acid) and appropriate tests demonstrated that TIBA effectively disrupted the polar auxin-transport system.

The authors wish to express their appreciation to Professor Ralph H. Wetmore for his interest in the study and for providing space and facilities in the Biological Laboratories, Harvard University and to Dr. Robert Z. Callaham of the Institute of Forest Genetics, Placerville, California, for his generous cooperation in supplying the seed. We also gratefully acknowledge the technical assistance of Miss Jane Nelson.

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CARBOHYDRATE CONTENT OF THE ROOT TIP CELLS OF ALLIUM CEPA[1]

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The first 2 mm of the root tip of *Allium cepa* have recently been analyzed for cell morphology (4) and the deoxyribosenucleic acid, ribosenucleic acid, and protein content of the cells (3). A similar analysis of the first 3 mm of the root tip of *Vicia faba* has also been reported (2) which included determinations of the carbohydrate and glucose content of the cells. These determinations indicate that with the beginning of elongation the increase in glucose-containing polysaccharides was not proportional to the increase in total carbohydrates. To determine if a similar condition exists in onion, the root tip cells of *A. cepa* were analyzed for total carbohydrate and total hexoses. The results of these determinations are presented here.

Roots were obtained from bulbs of *A. cepa* var. White Globe, which had been placed in vials of tap water. The roots, 10 to 15 mm in length when collected, were frozen-dried, infiltrated with paraffin, imbedded, and sectioned at 50 μ. Two 50-μ sections were placed in a 10×50 mm test tube thus equaling

[1] Received September 10, 1957.

a 100-μ section of tissue. The first twenty 100-μ sections of tissue were used in the analysis. As the analyses required more than 100 μ of tissue, as many additional roots as necessary were cut in a similar manner and the corresponding sections placed in the same tubes. The paraffin was removed with toluene and the tissue rinsed with 100 % ethyl alcohol.

Total carbohydrates were measured by the method of Sørenson and Haugaard as given by Jensen (2). This is an orcinol reaction equally sensitive to hexoses and pentoses. Standard curves were run on both glucose and xylose although the results are expressed as "glucose equivalents." Three roots were necessary for a determination and 6 sets of determinations were made.

Total hexose was measured by a modification of the method of Dische et al (1). The tubes containing the tissue were cooled in ice water and 0.5 ml cold H_2SO_4 (6 vol conc H_2SO_4 to 1 vol H_2O) added. After a few minutes the tubes were brought to room temperature and placed in boiling water for three minutes. After cooling 0.1 ml of a freshly prepared 3 % aqueous solution of cysteine was added and the mixture shaken. The color was read after two hours at 415 mμ and 380 mμ, the difference being a quantitative measure of hexoses. Glucose was used as the standard. Six roots were necessary for a determination and 6 sets of determinations were made. Because of the steps involved in introducing and removing the paraffin, it is assumed that most, if not all, soluble sugars have been lost before the measurements were made and that the cell wall is the major cellular component determining the results.

The amount of total carbohydrate and total hexose per cell is presented in figure 1. The results from each section are plotted at the mid point of the section analyzed from the tip, i.e., the data of the section between 200 and 300 μ from the tip is plotted at 250 μ from the tip. The small mass of tissue contained in the first two 100-μ sections made it impossible to obtain satisfactory data from them.

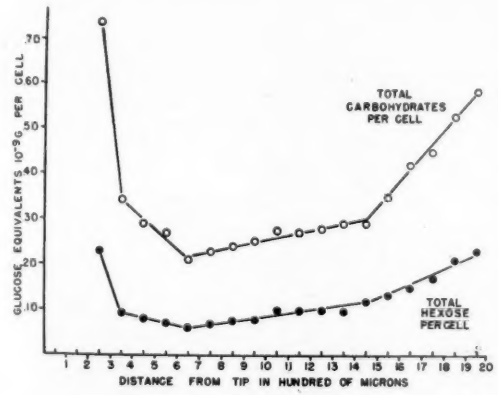

FIG. 1. The amount of total carbohydrate and total hexose per cell of the root tip of *Allium cepa* as a function of the distance of the section analyzed from the tip of the root.

The high values at the tip of the root indicate a high carbohydrate and hexose content for the root cap cells, probably as a result of their heavy cell walls. These heavy cells walls are plainly evident upon histological examination. When the meristem is reached at 450 μ from the tip, the decrease of value per cell is a result of the presence of cells other than the root cap, and these cells probably have a low carbohydrate content. By 650 μ from the tip the root cap cells no longer constitute a significant proportion of the cells in the section and the remainder of the figures are values of cells other than the root cap, primarily cortex and epidermis. The carbohydrate content of these cells increases steadily between 650 and 1550 μ from the tip. From 1550 μ on, carbohydrate content increases at a much greater rate. This pattern of increase in total carbohydrate content per cell from 650 μ on, is identical with the pattern of increase in cell volume for the cortical cells in the same area (4). Total hexose follows this same pattern except that the rate of increase from 1550 μ does not keep pace with the rate of increase in total carbohydrate and cell volume. This is in agreement with the data obtained from *V. faba* root tip (2) where it was found that total carbohydrate and total glucose increased at the same rate until the beginning of cell elongation. The rate of glucose increase then dropped markedly below that of the total carbohydrate.

SUMMARY

The total carbohydrate and total hexose content of the cells of the first two millimeters of *Allium cepa* were determined. The data indicate that total carbohydrate content of the cells is directly proportional to cell volume. This is also true for total hexose content during the early stages of development. With elongation however the increase in total hexose is not directly proportional to increase in cell volume suggesting the formation of a non-hexose containing cell wall component.

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MALATE SYNTHESIS IN CRASSULACEAN LEAVES. I. THE DISTRIBUTION OF C^{14} IN MALATE OF LEAVES EXPOSED TO $C^{14}O_2$ IN THE DARK[1]

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In recent years considerable interest has focused on the problem of the marked diurnal fluctuation of acidity which occurs in the leaves of certain plants, notably those of the Crassulaceae. Contributions from a number of sources have established or confirmed the following facts:

1) In the dark, after a period of strong illumination, detached leaves of Kalanchoë, Bryophyllum and some other plants undergo marked increases in free acid (1) associated primarily with increases in malic acid (2, 3, 4).

2) The accumulation of malic acid is accompanied by the fixation of CO_2 in amounts easily measured by classical techniques (5, 6, 7), and this accumulation is closely related to the partial pressure of CO_2 in the surrounding atmosphere (8, 7).

3) When $C^{14}O_2$ is supplied to the darkened leaves, radioactivity appears primarily in the organic acid fraction (9, 10) and, of the organic acids, malic acid becomes most heavily labeled (10, 7).

4) β-Carboxylase activity has been demonstrated in partially purified extracts of the leaves of Kalanchoë (11, 12) and of Bryophyllum (13, 14) and both malic enzyme and phosphoenolpyruvic (PEP) carboxylase have been detected.

If as appears probable (15, 16, 17), the PEP-carboxylase catalyzes the massive CO_2 fixation which occurs in Crassulacean leaves, the initial product of CO_2 fixation would be oxalacetic acid (OAA):

$$PEP + CO_2 \longrightarrow OAA + \text{inorganic phosphate}.$$

The malic dehydrogenase activity of these tissues, however, is more than adequate to account for the rapid conversion of OAA so formed to the malic acid which accumulates (12).

As a result of their study of $C^{14}O_2$ fixation by young Bryophyllum leaves over very short periods, Saltman et al (14) have suggested that OAA is in fact the first product of CO_2 fixation in these leaves.

We have been interested in the distribution of label in malic acid after various periods of exposure of mature leaves of Kalanchoë and Bryophyllum to $C^{14}O_2$ in the dark. In this paper experiments are described and results from a number of sources are collated which suggest that a preliminary carboxylation reaction may precede the β-carboxylation step, and that oxalacetic acid may itself arise from a labeled precursor.

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EXPERIMENTAL METHODS

Mature leaves of *Kalanchoë crenata* and *Bryophyllum calycinum* were cut in the late afternoon and usually given a further period of illumination to ensure vigorous acidification in the subsequent dark period. $C^{14}O_2$ was generated from $BaC^{14}O_3$ and supplied to the leaves after they had been in the dark for at least 30 minutes. For long term studies (5 minutes to 24 hours) entire leaves were enclosed in gas jars which were darkened and maintained in a bath at the desired temperature. The jars were then partially evacuated and $C^{14}O_2$ was swept in from an adjacent reservoir. After the desired exposure the leaves were killed by dropping them into boiling 80 % ethanol. For the short term studies disks approximately 1 cm in diameter were cut from a number of leaves with a sharp cork borer. The disks were enclosed in a small darkened chamber and $C^{14}O_2$ was swept in from the adjacent reservoir under slightly reduced pressure (fig 1). Removal of the residual $C^{14}O_2$ and killing of the leaf disks by infiltration with boiling 80 % ethanol were accomplished within one second by the rapid evacuation of the leaf chamber and introduction of the solvent. After killing, the leaves and leaf disks were exhaustively extracted with 80 % ethanol and water.

Malic acid was isolated chromatographically on Whatman No. 1 or No. 3 paper, with *tert*-amyl alcohol : formic acid : water (3 : 1 : 3) and propanol : ammonia : water (3 : 1 : 1) as the developing solvents (see, e.g., 18).

The malic acid ($\overset{1}{C}OOH\text{-}\overset{2}{C}HOH\text{-}\overset{3}{C}H_2\text{-}\overset{4}{C}OOH$) isolated was degraded by a variety of procedures. Carbon-4 was removed as CO_2 by *Lactobacillus arabi-*

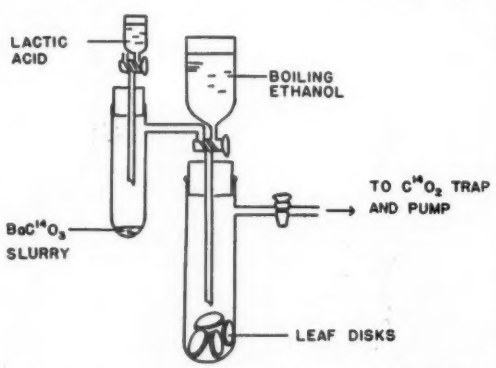


FIG. 1. Assembly for short term exposure of leaf disks to $C^{14}O_2$ in the dark.

nosus in a modification of Nossal's method (19). In some cases the residual lactate was isolated chromatographically and further degraded with acid permanganate, according to the method of Katz, Abraham and Chaikoff (20). This procedure yields C-1 of malate as CO_2 and C-2 + C-3 as acetic acid. The latter was recovered by steam distillation. In other experiments the lactate was degraded by the procedure of Friedemann and Kendall (21) to give CO_2 from C-1 and acetaldehyde from C-2 + C-3. Malate was also totally decarboxylated by this method to give the same products. In both instances acetaldehyde was trapped as its 2,4-dinitrophenylhydrazone.

The CO_2 arising from the carboxyl groups was collected as $BaCO_3$ and plated on either aluminum or sintered porcelain disks. When lactate was not further degraded it was precipitated as calcium lactate and plated as such on aluminum disks; acetaldehyde dinitrophenylhydrazone and sodium acetate were plated directly. For these compounds it was assumed that the self absorption was not appreciably different from that of $BaCO_3$ (see, e.g., 22). The radioactivity was assayed with a conventional end window Geiger-Müller tube and all counts were corrected for background and self absorption.

Results and Discussion

The percentage distribution of isotope within the malic acid isolated in a representative selection of experiments is recorded in table I. It can be seen that the label appeared in C-1 of malate after the shortest measured fixation time (4 seconds). Furthermore, a similar distribution of label in malate was found after all other fixation periods. In a wide range of times and temperatures, C-1 always contained approximately one third of the tracer fixed in malate. No C^{14} was detected in C-2 and C-3 of the malic acid isolated in any experiment.

Table II records the results of experiments in which the initial period of dark fixation was followed by exposure to darkness or to light, after the removal of residual $C^{14}O_2$ from the surrounding atmosphere. It will be seen that in all instances the radioisotope was confined to the carboxyl groups, with one third of the activity occurring in C-1. It would appear, therefore, that the distribution of radioactivity in malate which was established during the actual period of exposure to $C^{14}O_2$ persisted for a considerable length of time, either in the light or in the dark.

Table II

Distribution of Label in Malic Acid When the Initial Period of Dark $C^{14}O_2$ Fixation by Kalanchoë Leaves Was Followed by Exposure to Light or to Darkness

Fixation time and temperature	Subsequent treatment	C^{14} distribution $^{1}COOH\text{-}^{2}CHOH\text{-}^{3}CH_2\text{-}^{4}COOH$		
9 hr, 19° C	None	37	0	63
9 hr, 19° C	17 hr, dark, 19° C	37	0	63
9 hr, 19° C	145 hr, dark, 19° C	37	0	63
3 hr, 10° C	None	32	0	68
3 hr, 10° C	3 hr, light, 25° C	36	0	64

* Expressed as percent of totals ranging from 3,000 to 13,000 cpm.

The most salient feature of the results is the emergence of a virtually constant distribution of label in the malic acid molecule. About one third of the tracer fixed in malate was found in C-1 after only 4 seconds, and this distribution persisted throughout a variety of experimental treatments. These include fixation at 10° C, a favorable temperature for rapid acid accumulation (7), as well as at higher temperatures, where the accumulation is less pronounced. Moreover, the observed distribution was maintained long after the cessation of the net accumulation of acid, which, according to Thomas and Ranson (7), is essentially complete in the first 12 hours of darkness. Even in the light, when consumption of acid is rapid (1), there was no change in the previously established labeling pattern.

In table III are recorded the results of a number of other workers, for Crassulacean leaves and a variety of other tissues. Again it will be observed that in all experiments with Kalanchoë leaves (23) the C^{14} is confined to the carboxyl groups of malate, and

Table I

Distribution of Label in Malic Acid after Various Periods of Dark $C^{14}O_2$ Fixation by Kalanchoë and Bryophyllum Leaves

Date	Plant material	Experimental treatment	C^{14} distribution * $^{1}COOH\text{-}^{2}CHOH\text{-}^{3}CH_2\text{-}^{4}COOH$		
June '57	Kalanchoë	4 sec, 20° C	37	0	63
Sept. '56	Kalanchoë	5 min, 18° C	39	0	61
June '54	Kalanchoë	90 min, 19° C	30	0	70
May '56	Kalanchoë	3 hr, 10° C	30	0	70
July '54	Bryophyllum	16 hr, 20° C	34	0	66
July '56	Bryophyllum	20 hr, 23° C	32	0	68
Sept. '52	Kalanchoë	21 hr, 20° C	39	0	61

* Expressed as percent of totals ranging from 600 to 20,000 cpm.

TABLE III

DISTRIBUTION OF LABEL IN MALIC ACID FROM A NUMBER OF TISSUES AFTER $C^{14}O_2$ FIXATION

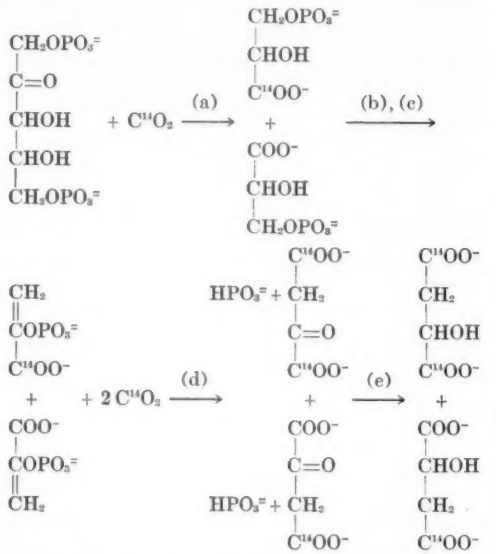
PLANT MATERIAL AND REFERENCES	FIXATION TIME AND TEMPERATURE	C^{14} DISTRIBUTION $^{1}COOH\text{-}^{2}CHOH\text{-}^{3}CH_2\text{-}^{4}COOH$		
Kalanchoë leaves (23)	2 hr, 10° C	38	0	62
	3 hr, 10° C	33	0	67
	9 hr, 10° C	26	0	74
	12 hr, 10° C	30	0	70
	24 hr, 10° C	33	0	67
Fumitory leaves (24)	5 min, 20° C	4	0	96
Sunflower seedlings (25)	45 sec, 24° C	12	0	88
	3 hr, 24° C	5	0	95
	12 hr, 24° C	5	0	95
Kalanchoë roots (23)	1 hr, 20° C	51	0	49
	6 hr, 20° C	48	0	52

that, with one possible exception, the ratio of C^{14} in C-4 to C^{14} in C-1 is of the order 2 to 1. In sharp contrast are the results obtained with the other tissues, namely leaves of fumitory (*Fumaria officinalis*) (unpublished data), seedlings of sunflower (*Helianthus annuus*) (24) and roots of Kalanchoë (*Kalanchoë crenata*) (23). None of these tissues shows the large diurnal fluctuation of acidity which is characteristic of Crassulacean acid metabolism, and, as table III indicates, neither do they show the constancy of the distribution of label in malate which has been observed in the Crassulacean leaves. It is of interest, therefore, to examine how leaves like those of Kalanchoë can synthesize large quantities of malate in the dark while maintaining in tracer experiments the asymmetric distribution of C^{14} in the two carboxyl groups.

If malate is produced, as seems likely, by a β-carboxylation mechanism, label would appear first in C-4. Presumably, if malate labeled in this way were subject to the randomizing action of fumarase, the label would spread from C-4 to C-1. But only if exactly two thirds of the malate molecules passed through fumarate prior to accumulation (as, for example, in the vacuole) would the 2 to 1 ratio be realized in the carboxyl groups of the accumulated malate. Alternatively, label fixed in C-4 might spread to C-1 through the action of the enzymes of the glyoxylic bypass (25). If exactly one third of the malate molecules were subject to the reactions of the bypass prior to accumulation, a 2 to 1 ratio might also be expected.

Clearly, the emergence of the observed ratio by either of these two mechanisms would seem to depend upon a very precise and closely maintained relationship between the rates of the various contributing reactions. Moreover, the results demand that this relationship should persist under widely varying conditions of time and temperature. Now, although the existence of such a relationship at a particular time and temperature is not entirely inconceivable, its maintenance in independence of the level of malate in the leaf, especially at different temperatures, seems most unlikly.

One mechanism (26, 27) which, however, would lead to malate synthesis with the required constant asymmetric distribution of label is that outlined below.



According to this mechanism, malate would be synthesized from a labeled 3-carbon precursor, 3-phosphoglyceric acid (PGA), produced from ribulose diphosphate, as it is in photosynthesis (28), by the action of ribulose diphosphate carboxylase (reaction a). When $C^{14}O_2$ is fixed in this reaction, labeled and unlabeled molecules of PGA arise in equal quantities; malate would be produced from this PGA through the successive action of phosphoglyceromutase (reaction b), enolase (reaction c), PEP-carboxylase (reaction d) and malic dehydrogenase (reaction e). As a result, label would appear in both carboxyl groups of malate, and the distribution of label between the two carboxyl groups would be in accord with that observed experimentally.

On the basis of this hypothesis, the asymmetry of the labeling in the malic acid molecule would be imposed at the time of its synthesis, rather than in subsequent randomizing reactions. Consequently it would be independent of time and temperature as the results in fact require. It is therefore suggested that malate synthesis in the Kalanchoë and Bryophyllum leaves studied occurs, in the main, by a pathway involving two carboxylation reactions, similar to that outlined above.

The proposed mechanism has a further implication for the metabolism of leaves of the Crassulacean type. Such leaves can easily accumulate as much as 500 mg of acid per 100 g fresh weight in the course of 12 hours (7). It is clear, therefore, that if the suggested

scheme for malate synthesis operates in these leaves, they must have a means for the provision of commensurate amounts of ribulose diphosphate in the dark. It is tentatively suggested that the reactions of the pentose phosphate pathway of carbohydrate breakdown (see, e.g. 29) might provide ribulose phosphate, which, upon phosphorylation, would serve as the initial CO_2 acceptor.

Some evidence for the participation of these reactions has already been found (30); in particular, it has been possible to observe the appearance of label in PGA during short term (4 second) dark fixation of $C^{14}O_2$ by Kalanchoë leaf disks. Further information is at present being sought from the appearance of specific labeling patterns in malic acid after the feeding of specifically labeled sugars and intermediates of the pentose phosphate pathway in the dark.

Summary

1. The distribution of label in the malic acid synthesized by mature leaves of Kalanchoë and of Bryophyllum during exposure to $C^{14}O_2$ in the dark has been determined.

2. It was found that the C^{14} was confined entirely to the carboxyl groups and that C-1 always contained approximately one third of the activity.

3. The above distribution was not affected by the duration or temperature of fixation or by subsequent exposure of the leaves to light or darkness in the absence of $C^{14}O_2$.

4. A mechanism which accounts for the observed constant asymmetry of labeling, and which involves the prior carboxylation of ribulose diphosphate is suggested, and some of its implications are considered briefly.

We wish to thank Professor M. Thomas, F.R.S., for his continued interest in this work, and also Dr. H. Beevers and Dr. D. A. Walker, for much valuable discussion and advice. This work was completed while one of us (M.S.) held a National Science Foundation Fellowship.

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EFFECT OF TRIIODOBENZOIC ACID AND MALEIC HYDRAZIDE UPON THE TRANSPORT OF FOLIAR APPLIED CALCIUM AND IRON[1,2]

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The lack of mobility of foliar applied calcium in a downward direction has been reported (1, 2, 3, 7, 8) and it has been suggested that polarity might play a role in this movement (3). By suspending polarity by mild anesthesis (10) with di-ethyl-ether the immobility of calcium was indeed overcome and calcium was translocated from the site of application on the leaves down to the roots (3).

These reports are of considerable importance with regard to the increasing use of foliar sprays. It was, therefore, of interest to study the effect of substances, which are believed to interfere with the polar movement of auxins, upon the transport of immobile elements. Such a substance, triiodobenzoic acid (TIBA) (5, 6), was, therefore, chosen for these experiments. For purposes of comparison a second growth inhibitor, maleic hydrazide (MH), was also tested.

Tomato plants and one-year-old apple trees were sprayed with either a 100-ppm solution of TIBA or a 100-ppm solution of MH, each solution containing 0.05 % of a spreader (TRITON B–1956). One terminal leaf of each plant was then treated with 0.1 ml of Ca^{45} (0.8 μc per ml). The Ca^{45} was dropped on the leaf surface with a micropipette and was absorbed there quickly. The same amount of radiocalcium was applied to plants which had received no TIBA or MH pretreatment, but only a spray with water containing the same amount of the spreader as above. A few days after the application of Ca^{45}, leaves located below the treated ones were collected from each apple tree, while from the tomato plants the lower part of the stem was sampled. These samples were ashed, and the radioactivity counted in a thin end-window Geiger-Mueller counter.

Results are presented in table I, and they confirm the limited transport of calcium in a basipetal direction. They further demonstrated the considerable promotive effect of TIBA on the downward transport of this element. MH on the other hand was much less effective in this respect.

TABLE I

EFFECT OF TIBA AND MH UPON THE BASIPETAL MOVEMENT OF Ca^{45} IN APPLE TREE SEEDLINGS AND TOMATO PLANTS

SPECIES	PART OF PLANT TESTED	TREATMENTS (COUNTS/MIN × G FRESH MATTER)		
		Ca^{45}	$MH + Ca^{45}$	$TIBA + Ca^{45}$
	leaf no. below treated leaf			
Apple	3	30	136	21,930
(5 days after	5	0	64	21,273
treatment)	7	0	52	500
	9	0	40	460
	20	0	0	117
Tomato	lower part			
(2 days after	of stem	0	...	2,000
treatment)				

A similar effect of TIBA was found upon the transport of iron, which is also frequently immobile. No radioactive material was employed in the experiments with iron. The effect of TIBA and MH upon the transport of iron was followed by the synthesis of chlorophyll in chlorotic leaves of peach and plum trees growing in high-lime soils. Iron sulphate, when sprayed on such chlorotic leaves, produces only localized green spots of chlorophyll (fig 1) as compared to a solid green surface when pretreated with TIBA, i.e., TIBA caused the iron to spread inside the leaf from the point of penetration, thus becoming available to all leaf parts for chlorophyll synthesis.

[1] Received September 16, 1957.

[2] Publication of the Agricultural Research Station, Rehovot, 1957 Series, No. 210-E.

Quantitative data on the effect of TIBA upon the transport of iron and its availability for chlorophyll formation were obtained by analysis of the chlorophyll content of the leaves. Chlorotic peach and plum trees were sprayed with 100-ppm solutions of TIBA and MH respectively. The pretreated plum trees were then sprayed with a 0.5 % solution of iron sulphate. In case of the pretreated peach trees the additional iron spray was not applied to the whole trees (as with plum trees), but only to the basal part of branches (5 branches per treatment). For analysis leaves from the terminal part of these branches were sampled, at a distance of about 30 to 40 cm from the treated leaves. For comparison leaves were collected from branches which received TIBA and MH pretreatments but no iron treatments, as well as leaves which received an iron spray with no TIBA and MH pretreatments. For chlorophyll determinations the leaves were extracted with ethanol, and the extracts compared with a bichromate-copper standard (4). The results presented in table II give indirect evidence of increased transport of iron under the influence of TIBA.

It is seen from table II that TIBA alone has a certain promoting effect upon the formation of chlorophyll. This promoting effect may be brought about by a freeing of immobilized iron by TIBA. A similar mechanism was indicated already on the basis of results obtained by Weinstein et al with chelates (9).

The introduction of iron into TIBA pretreated leaves caused a considerable increase in chlorophyll formation, as compared with the effect of TIBA alone. Based on the above results (fig 1) it can be suggested that TIBA enables the penetrating iron to spread inside the leaves, keeping the iron from becoming immobilized and thus remaining available for chlorophyll synthesis. The experiment with peach trees shows further that TIBA promotes also, similarly to calcium, the transport of iron over appreciable distances, from the site of application at the basal leaves to the more remote apical leaves.

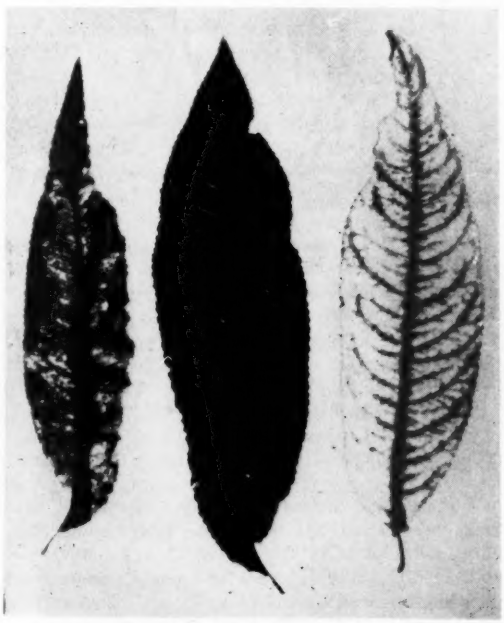

FIG. 1. The effect of TIBA upon the distribution of externally applied iron in chlorotic peach leaves, as measured by the formation of chlorophyll. Left: Fe without pretreatment with TIBA; center: Fe, pretreated with 100 ppm of TIBA; right: control (no treatment).

TABLE II

EFFECT OF TIBA AND MH UPON THE TRANSPORT OF IRON IN CHLOROTIC PEACH AND PLUM LEAVES AS MEASURED BY THE SYNTHESIS OF CHLOROPHYLL 35 DAYS AFTER TREATMENT

TREATMENTS	PEACH * VAR. C. O. SMITH	PLUM ** VAR. KELSEY
Control	0.129	0.078
MH	0.136	....
TIBA	0.151	0.193
Fe	0.289	0.132
Fe + MH	0.254	....
Fe + TIBA	0.463	0.351
Standard error	0.008	0.019

* Chlorophyll percent of fresh matter of leaves distal to the leaves which received an iron spray.

** Chlorophyll percent of fresh matter of leaves which received an iron spray.

MH, on the other hand, had no effect upon the transport of iron (as indicated by the lack of chlorophyll synthesis), although it had, however only limited, an effect on the transport of calcium.

Under conditions of similar concentrations of TIBA and MH it is as yet difficult to say whether the effects of these substances differ only quantitatively at the level used in these experiments, or whether different mechanisms are involved through which these substances affect the plant.

The above results are of special interest with regard to TIBA as a growth inhibitor. The mobility and downward transport of nutrients might well be related to the suggested suspending effect of TIBA upon polarity, its interference with the polar movement of auxin, and its destroying effect upon apical dominance.

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THE ACTION SPECTRUM FOR PHOTOSYNTHETIC PHOSPHORYLATION BY SPINACH CHLOROPLASTS[1,2,3]

ANDRÉ T. JAGENDORF, STERLING B. HENDRICKS, MORDHAY AVRON AND MARJORIE B. EVANS

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Photosynthetic phosphorylation by chloroplasts was first described by Arnon et al (1). It is believed to involve the following reactions:

$$1.\ H_2O \xrightarrow[\text{chloroplasts}]{\text{light}} 2(H) + (O)$$

$$2.\ ADP + P_i + 2n\ (H) + n\ (O) \rightarrow nH_2O + ATP$$

Reaction 1 is presumably the only reaction where light is involved in photosynthesis, in the Hill reaction, and in photosynthetic phosphorylation. Under this hypothesis the action spectra of the three processes should be identical. It has previously been shown that the action spectra for photosynthesis and for the Hill reaction do indeed correspond to each other and to the absorption spectrum of chlorophyll (2). It seemed of interest to determine whether the action spectrum for photosynthetic phosphorylation would similarly relate to the absorption spectrum of chlorophyll.

The experiments were performed with the high irradiance spectrograph of the U. S. Department of Agriculture at Beltsville, Maryland. Chloroplasts and the reaction mixture were prepared as previously described (3) with phenazine methosulfate and magnesium as the only cofactors. Phenazine methosulfate has an absorption peak at 388 mμ, and only very slight absorption above 400 mμ. It has previously been found (3) that photosynthetic phosphorylation

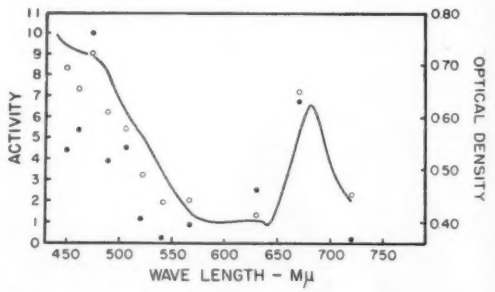

Fig. 1. Action spectrum for photosynthetic phosphorylation. The solid line represents the absorption spectrum of the chloroplast suspension (scale on right). The points represent the activity of the preparation expressed as micromoles phosphate taken up per mg chlorophyll per hour per milliwatt/cm^2 of light energy at the particular wave length (scale on left). The solid circles were obtained in one experiment, with .114 mg chlorophyll per 25-ml Erlenmeyer flask; the open circles from a 2nd experiment with 0.076 mg chlorophyll per flask. One and five-tenths ml of reaction mixture were spread over the bottom surface of approximately 10 cm^2. The flasks were placed in a rectangular, glass bottom, Warburg bath kept at 15° C and were shaken continuously at a small amplitude. The light was directed at the flask bottoms with the aid of a mirror. Reactions were run under N_2 for 30 to 60 minutes.

The reactions represented in this figure were run with the spectrograph set as a single prism instrument, with the slit width equal to 1.5 times the separation of wave length positions of adjacent flasks between 450 and 570 mμ.

[1] Received September 19, 1957.

[2] Contribution No. 202 from the McCollum-Pratt Institute.

[3] Supported in part by grant RG-3923 from the National Institute of Health, Research Grants Division.

was not saturated with white light up to an illumination of 4,000 ft-c, and was a linear function of light intensity below that point. Since the intensity of light available from the spectrograph (0.4 to 2.0 milliwatts/cm^2) was very much lower than the saturation intensity, the reactions can be assumed to have been run at the linear portion of the activity vs. intensity curve.

With the light intensity so severely limiting, the rate of the reaction is not directly proportional to the concentration of chloroplasts. As a result activity values of experiments run with different amounts of chloroplasts are not strictly comparable. Within each experiment the amount of chloroplasts per flask was the same at every station across the spectrum. In order to make relatively small amounts of phosphate taken up perceptible with the method used, the total amount of phosphate in the flask was decreased to 0.83 micromoles/ml. The reaction was measured by following the disappearance of inorganic phosphate from the medium. The amount of energy available at each wave length was measured with the aid of a thermopile at flask height.

Figure 1 illustrates the action spectrum obtained when the flasks were located across the visible spectrum. The solid line in this and the following figures represents the absorption spectrum, with some loss due to scattering, of a similar chloroplast suspension as measured with a recording Beckman DK spectrophotometer. The experimental points represent two duplicate experiments. It is evident that red (approx. 680 mμ) and blue (approx. 475 mμ) light are most effective, and green light least effective in photosynthetic phosphorylation. The ratio of the blue to red peak values in different experiments varied between 1.2 and 1.8.

Better resolution was obtained by measuring the blue end and the red end separately. Figure 2 illustrates the action spectrum at the blue end of the spectrum. The agreement between the absorption spectrum and the action spectrum is quite good. The shoulder in both cases at approximately 480 mμ indicates that carotenoid pigments can be primary light receptors. This has previously been shown in action spectra of photosynthesis and the Hill reaction (3, 4).

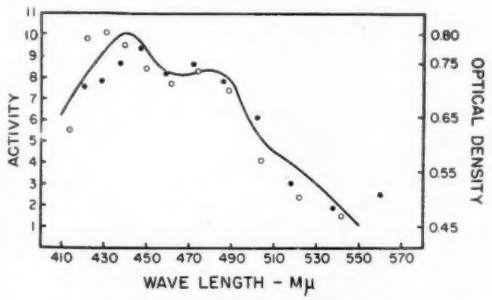

FIG. 2. Blue region of the action spectrum for photosynthetic phosphorylation. Details as in figure 1. Two different experiments are shown, both with .061 mg chlorophyll per flask.

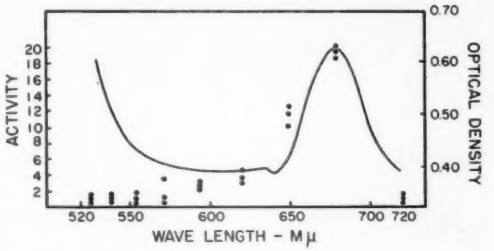

FIG. 3. Red region of the action spectrum for photosynthetic phosphorylation. Details as in figure 1, except that the spectrograph was set as a double prism instrument, and the slit width was equal to the difference of wave length positions of adjacent flasks. Three different experiments are shown, each with .081 mg chlorophyll per flask.

The action spectrum at the red end of the spectrum is shown in figure 3. To obtain better resolution in the red, the spectrum was spread for these measurements. Here, again, the agreement with the absorption spectrum is quite good at the red peak. The absorption of the chloroplast suspension between 520 and 550 mμ seems to be due to pigments ineffective as light receptors for photosynthetic phosphorylation.

In conclusion, within the limits of measuring procedure, the action spectrum for photosynthetic phosphorylation resembles closely the absorption spectrum of the chloroplast suspension, of chlorophyll, and the action spectra for both the Hill reaction and photosynthesis. It lends further support to the theory that all three processes possess the same initial light reaction.

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American Society of Plant Physiologists, *Executive Committee Meeting, Stanford, California, August 25th, 1957:* The Executive Committee of the society met in room 380 of the Outer Quadrangle on the campus of Stanford University. The meeting was opened at 7:10 by President Folke K. Skoog. Present were: W. D. Bonner, Jr., H. A. Borthwick, A. H. Brown, A. W. Galston, D. R. Goddard, W. C. Hall, P. J. Kramer, W. E. Loomis, E. H. Newcomb (substituting for R. M. Muir), G. R. Noggle, F. K. Skoog, and H. Stout. Present to present special reports were: M. W. Parker and F. C. Steward.

Reading of the minutes was omitted because of their prior publication in the January issue of *Plant Physiology*. President Skoog reported that the society continues to progress, both financial and editorial matters continuing in a satisfactory condition. Membership in the society and subscriptions to the journal are continuing the upward trend noted last year.

The Secretary reported on several matters. 1) The elections resulted in the following new slate of officers: President, David R. Goddard, University of Pennsylvania; Vice-president, Arthur W. Galston, Yale University; Secretary, Walter D. Bonner, Jr., Cornell University; Member of the Executive Committee, James Bonner, California Institute of Technology; Members of the Editorial Board, Harry A. Borthwick, U.S.D.A., Plant Industry Station, Beltsville, Maryland, and Theodore C. Broyer, University of California, Berkley. 2) Five hundred and seventy-six members of the society cast their ballots in the final election, once again approximating a 50% voting record. The new officers will begin their duties as of January 1, 1958. 3) The abstracts of the papers scheduled for presentation at the annual meetings were once again mailed early in July and reached the membership at least one month in advance of the meeting. The abstracts, published as a supplement to Volume 32 of *Plant Physiology*, continue to cost about 40 cents per member, exclusive of mailing costs. Despite the printing of 3,100 abstracts, there are none available for sale this year as increased subscriptions and membership have resulted in a great drain on the reserve. There seems general agreement that the society should continue to prepare the abstracts as they have done in the last three years. 4) In the past, the home institution of the Secretary has absorbed all the clerical costs for the preparation of these abstracts. Because of the early deadline after the receipt of the manuscripts from authors, it is often necessary to obtain extra help to complete this intensive work within the deadline. It was moved by D. R. Goddard and seconded by A. W. Galston that the President may empower the Secretary to make a modest expenditure to aid him in the preparation of these abstracts. The motion passed unanimously. 5) At the request of President Skoog the business meeting of the society, usually held in conjunction with the annual banquet, will be transferred to a separate session. This will be held immediately after the Tropism Symposium on Monday afternoon at 4:30. This should make it possible to schedule exclusively pleasurable events for the banquet. 6) There continued to be requests throughout the year for vocational guidance literature. At the present time, the Secretary sends to each person requesting information two items, "Plant Physiology as a Career" published by our society in 1954 as a result of the work of the Committee on Professional Status and Training under the chairmanship of James Bonner, the "Career Opportunities in Biology" prepared for the National Academy of Sciences, National Research Council, under the editorship of Russell B. Stevens. The supply of both these documents is running low. There was discussion of the desirability of publishing large numbers of reprints of the new report of the Committee on Professional Status and Training under the chairmanship of Victor A. Greulach. Dr. Goddard reported that the entire report would appear in *Plant Physiology* and suggested that each member of the 6-man committee receive 25 reprints at the society's cost. It was felt that this report, being highly technical, is not suitable for vocational guidance literature and that a separate report should probably be made, perhaps by another committee, to serve as up-to-date vocational guidance information. 7) There was a discussion of the role of sections in the life of the society. In view of the fact that the national meetings are becoming increasingly crowded, the Secretary felt that some change in form might become necessary and that the contribution of papers by individual members might be more suitable at the sectional meetings of the society. There was general agreement, however, that at the present time there was no need for any drastic change in format of the national meetings. Each section is to operate autonomously, scheduling whatever meetings it desires. The Secretary emphasized that the section secretaries should inform the national secretary of their activities so that these could be called to the attention of the entire membership. The Committee felt that abstracts of the papers presented at sectional meetings should be prepared for the entire membership, the national secretary rendering aid as required. It is paradoxical but true that individuals may be members of a section without being members of the national society. This obviously makes impossible any tight control of the sections by the national society.

The Editor of *Plant Physiology*, Dr. D. R. Goddard, presented a report emphasizing the growing quality and vigor of the journal.

Papers received (August 15, 1956 to August 13, 1957)	254
Papers accepted	172
Papers published	130
Papers for publication	42
Papers at press	16
Papers on hand	26
Papers withdrawn	7
Papers rejected	26
Papers for revision	23
Papers in process	26
Papers for decision	4
Papers being reviewed	15
New papers	7

Publication by issue:		Number of papers
1956	July	15
	September	20
	November	18
1957	January	16
	March	18
	May	19
	July	24
	Total	130

Comparative Study:	1954	1955	1956	1957
1. Total number of manuscripts received	157	150	151	254
2. Number printed	87	100	115	130
3. Number in press	39	40	26	42
4. Number accepted, not published	5	15	12	23
5. Number of manuscripts rejected or withdrawn	65	32	27	33
6. Number in process	29	12	29	26

Dr. Goddard will continue to work on the remainder of Volume 32 for the calendar year 1957. Dr. Allan H. Brown will be Editor as of Volume 33, and is already at work on manuscripts which have been received too late for inclusion in Volume 32. All future manuscripts should be sent to Dr. Allan H. Brown, Editor, *Plant Physiology*, Department of Botany, University of Minnesota, Minneapolis 14, Minnesota. The members of the Executive Committee warmly thanked Dr. Goddard for his great service in improving the journal over the last few years.

The Executive Secretary-Treasurer, Dr. G. R. Noggle, presented a prepared report on the financial status of our society.

Membership:	July 1, 1956	July 1, 1957
Life members	34	34
Corresponding	19	19
Canadian	39	38
U. S. members—regular	1014	1002
student	....	79
Puerto Rico, T. Hawaii and Alaska	28	27
Foreign members	110	122
	1244	1321
Less those included in other groups	5	5
	1239	1316

Subscription:				
U. S., Vol. 31	398	Vol. 32	426
Foreign, Vol. 31	890	Vol. 32	1093
	1288		1519

Summary of Cash Accounts:	
Checking account, July 1, 1956	$11,943.04
Savings account, July 1, 1956	17,000.00
Cash balance, July 1, 1956	$28,943.04
Receipts, 1956–57	36,461.53
Total	$65,404.57
Expenditures, 1956–57	22,524.59
Cash balance, July 1, 1957	$42,879.98
Cash on hand, July 1, 1957	
Checking account	$25,679.48
Reserve account	17,200.50
Total	$42,879.98

Summary of Endowment Account:	
(*Life Membership and General Endowment Fund*)	
Balance, July 1, 1956	$ 626.50
Income, 1956–57	
Sales of back numbers of journal	570.45
Investments	887.98
From Iowa State Press	
Subsidy and royalty on monographs	2,955.91
Royalty earnings "Growth and Differentiation"	1,114.51
Royalty earnings "Photosynthesis in Plants"	190.37
Total, balance and income	$ 6,345.72

Dr. Noggle's report was unanimously accepted and great satisfaction was expressed that he was able to assume the complicated duties of this office with so little difficulty.

Reports of standing and special committees and representatives followed. Dr. F. C. Steward, our representative to the National Research Council, reported on various matters which had been discussed during the year by that body. Among the topics mentioned briefly were: 1) aid to Hungarian refugees; 2) report on Radiation Biology, widely disseminated in the press and scientific literature; 3) international relations and the question of visas. Under this heading Dr. Steward reported that scientific meetings may now easily be scheduled in the United States. The State Department is willing to cooperate to see that foreign representatives from all countries receive visas for travel to an authorized scientific meeting. It was mentioned that had such a ruling been made earlier the International Botanical Congress scheduled for Montreal probably would have been held in the United States. 4) National Science Foundation fellowships. Dr. Steward reported that there were fewer acceptances in biology than in chemistry. The causes for this inequality are unknown. There continues to be inadequate botanical representation on the various boards choosing fellows, recipients of travel grants and national research grants. The Executive Committee felt it desirable to

communicate with the proper authorities on the National Science Foundation to correct this situation.

Dr. P. J. Kramer, our representative to the AIBS gave us the following report: 1) There are now 55,000 members in the AIBS, including 17,000 non-duplicated names in 21 member societies; 2) the annual budget of the AIBS is now in excess of $300,000 including about $200,000 in contract services to such organizations as the Office of Naval Research, National Science Foundation, etc.; 3) the AIBS has a vigorous program of visiting lecturers and has now assumed the editorship of the *Quarterly Review of Biology;* 4) various monographs are to be translated under AIBS auspices, especially from the Russian language. Members are requested to pass suggestions to the AIBS for suitable material to be translated. The Secretary remarked that one suitable project would be the translation of the German portions of the *Encyclopedia of Plant Physiology* into English for ready availability to graduate students. 5) The AIBS is now exploring the possibility of assuming many of the routine office and editorial functions of member societies. Under such a plan a central staff would be permanently employed to handle such matters for all societies desiring this service. Dr. Goddard was enthusiastic about such a possibility, saying that the life of the editor would be made much simpler by such a procedure. Dr. Brown moved that we cooperate in exploring the possibility of joint editorial and office services being supplied by the AIBS and that we advance money to send two delegates to a meeting to explore these possibilities. The motion was unanimously carried and Drs. A. H. Brown and G. R. Noggle were designated as our society's representatives.

The schedule of future AIBS meetings is as follows: 1958, Indiana University; 1959, Pennsylvania State University, after the International Botanical Congress in Canada; 1960, Stillwater, Oklahoma.

Dr. H. G. Gauch reported that there had been no important activities of the Agricultural Research Institute during the past year.

Dr. M. W. Parker, representative for the AAAS Council, reported that the suggestion of the Council for each member society to provide $25.00 toward a travel fund for members of the Council attending the annual meeting was not accepted by member societies. The Constitution of the AAAS has been amended. Associate societies have been abolished; all are now designated affiliates so as to have representation on the Council. Among the major activities of the AAAS for the past year have been: 1) establishment of the Committee of the Social Relations of Science; 2) subsidization of a contract between the AAAS and the International Cooperation Administration to facilitate the training of foreign nationals sponsored by the ICA; 3) continuation of the traveling library program under grants on the NSF; 4) dissolution of the Springfield chapter; 5) a conference on marine sciences; and 6) science information programs for the public. Aubrey W. Naylor of Duke University has been designated the new Secretary of Section G; Harry J. Fuller of the University of Illinois has been named Vice-president of Section G, and Paul E. Klopsteg has been reelected a member of the board of the AAAS.

Dr. M. A. Sprague, representative to the Joint Committee on Grassland Farming, reported that this group has developed a program of interest to plant physiologists. On August 29 a full-day program of papers has been arranged in conjunction with the Stanford meetings, and on August 30 a Grassland tour has been arranged to neighboring counties.

Dr. Greulach reported for the Committee on Professional Status and Training. The Committee currently consists of: H. M. Benedict, T. C. Broyer, V. A. Greulach (chairman), C. J. Lyon, H. B. Tukey, and C. H. Wadleigh. The Committee has recently completed its report on plant physiologists in industry. The preliminary report was submitted to the Executive Committee in 1955 and approved for publication. The final draft has now been submitted to the Editor of *Plant Physiology* and will shortly appear in that journal. Subsequent to the Storrs meeting the report was extensively revised, several people, including J. van Overbeek and R. F. Dawson having made detailed criticism. Since the entire study has been conducted at no expense to the society, the Committee suggests that the society bear the expense of having a suitable number of reprints prepared. Each committee member would like 25 reprints for a total of 150; and, in addition, 40 copies should be sent to the industrial research directors who answered the questionnaire upon which the report is based. The Committee further suggests that, if another study of this magnitude is to be made in the future, some grant be obtained to support it. One to two thousand dollars would probably suffice. The Committee has not begun its work on a new career booklet which the Executive Committee asked it to prepare at the Storrs meeting. Work will be done on this during the coming year. The Committee welcomes suggestions for persons who might be capable of writing such a report in cooperation with the Committee or independently. The Committee further suggests that immediate steps should be taken to implement a recommendation in the report that a Symposium on plant physiology in industry should be held in conjunction with an annual meeting, preferably the one in 1958.

Dr. Borthwick, our representative to the Editorial Board of the *American Journal of Botany* reported that he had had no duties in his post but considered the liaison to be important and would be happy to continue his service to the society.

Dr. van Overbeek, representative to the American Standards Association, reported on several activities of the Committee on Standards for Agricultural Chemicals and the Committee on Common Names for Pest Control Chemicals. He regards our continued affiliation with this group as important.

President Skoog accepted the recommendation of

our new Editor, Dr. Allan H. Brown, that Jack Myers of the University of Texas and Robert H. Burris of the University of Wisconsin be appointed to the Editorial Board of *Plant Physiology*. Other appointments were held in temporary abeyance.

Carlos O. Miller, who will shortly take up new duties at Indiana University in Bloomington, was designated by President Skoog as our local representative for the 1958 meetings. There was some discussion of a placement service for plant physiologists, an item which has recurred on our annual agenda for several years. Dr. Kramer reported that the AIBS is now prepared to assume this burden and the Executive Committee expressed general approval of this plan.

The following budget was approved for *Plant Physiology* for 1958:

Editor's stipend	$ 1,000
Editorial Assistant	2,500
Office supplies	300
Printing and publication costs	19,000
Contingency fund	300
Total	$23,000

There was some discussion of a proposal by L. W. Roberts that our society aid our Hungarian colleagues in restoration of their laboratories and libraries. The Executive Committee felt that our society could not undertake such activity and that proper representation be made to suitable granting agencies.

Dr. Noggle reported that a new directory should be prepared soon. The Executive Committee authorized its publication.

The First National Bank of Gainesville was designated as our bank for the handling of checking and saving accounts. Dr. Noggle also mentioned that considerable fluid funds are now available for investments. The Board of Trustees, which will shortly be augmented by an additional member, will be urged to make these investments as soon as possible.

President Skoog brought before the Committee the possibility of the printing of the abstracts of our meeting on microcards. This has been suggested by the AIBS as a service to societies. There was general feeling that the present abstracts would be superior and that no action be taken on the microcard suggestion. Dr. Skoog also mentioned that an organization of biology editors had been elected to the AIBS. He felt that this was a mistake but nothing can be done about it now.

The meeting was adjourned at 10:30.

ANNUAL BUSINESS MEETING: The meeting, held in Cubberly Auditorium on August 26th, was opened by President F. K. Skoog immediately after the conclusion of a Tropism Symposium. The Secretary reported on the meetings at Storrs, on the elections, and the abstracts. The innovations at the meetings, including the introduction of informal sessions and a round-table was discussed, as well as the separation of the business meeting from the banquet. The highlights of the Executive Committee meeting were briefly outlined and attention was called to the fact that the minutes will be published in the January 1958 issue of *Plant Physiology*. The Executive Secretary-Treasurer, Dr. Noggle, reported that our society was in good shape financially and that membership continued to increase. Dr. Goddard similarly reported that our journal, *Plant Physiology*, continues to improve in quality and in promptness. This calendar year 7 issues containing 130 papers will have been printed. Of the 254 papers received only 30 were rejected. Thirty are still in process. The last issue of this year will be an especially large one so as to clear the decks for the new Editor, Dr. Allan H. Brown. President Skoog expressed the appreciation of the entire society for the fine work accomplished by Dr. Goddard during his tenure of office as Editor.

The meeting was adjourned at 4:50.—Respectfully submitted, ARTHUR W. GALSTON, *Secretary*.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *appointments:* The following appointments have been made by President Skoog with the approval of the Executive Committee: Editorial Board of *Plant Physiology*, R. H. Burris and Jack Myers; Board of Trustees, S. B. Hendricks (reappointment); Representative to National Research Council, Robert Emerson, alternate, L. Blinks; Representative to Agricultural Research Institute, W. B. Howell, alternate, A. S. Crafts; Representative to AIBS Governing Board, B. S. Meyer, alternate, Anton Lang; Representative to the Editorial Board of *American Journal of Botany*, H. A. Borthwick (reappointment), alternate, J. G. Torrey; Representative to American Standards Association, Committee on Standards for Agricultural Chemicals, J. van Overbeek (reappointment); Representative on Joint Committee on Grassland Farming, M. A. Sprague (reappointment); and Representative to National Register of Scientific Personnel, D. B. Anderson.—ARTHUR W. GALSTON, *Secretary*.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Western Section:* New officers of the section are: Chairman, Noe Higinbotham, Washington State College; Vice-chairman, C. R. Stocking, University of California at Davis; and Secretary-Treasurer, John D. Spikes, University of Utah.—ARTHUR W. GALSTON, *Secretary*.

AMERICAN SOCIETY OF PLANT PHYSIOLGISTS, *Midwestern Section:* The section held its 3rd annual meeting at the University of Michigan, Ann Arbor, June 28th and 29th, 1957. On the morning of the 1st day 20 contributed papers were presented in two concurrent sessions. In the afternoon there was a symposium on *Structure and Function in Photosynthesis*, under the chairmanship of Dr. A. W. Frenkel. The morphology and activities of chloroplast preparations and of their bacterial counterparts were discussed. On the evening of the same day a banquet was held. Dr. R. M. Muir presided and Dr. Stanley Cain addressed the section members on the topic: The Tropical Rain Forest. Six round-table discussions occupied the morning of the 2nd day. In the afternoon there was a symposium on *Ion Metabolism* with R. S. Ban-

durski as chairman. The new officers of the section are: Chairman, Sam Aronoff, Iowa State University; Vice-chairman, Eldon Newcombe, University of Wisconsin; and Secretary-Treasurer, Alfred Sussman, University of Michigan.

IXth International Botanical Congress: The Ninth International Botanical Congress will be held in Montreal, Canada, from August 19 to 29, 1959, at McGill University and the University of Montreal. The program will include papers and symposia related to all branches of pure and applied botany. A first circular giving information on program, accommodation, excursions, and other detail will be available early in 1958. This circular and subsequent circulars including application forms will be sent only to those who write to the Secretary-general asking to be placed on the Congress mailing list: Dr. C. Frankton, Secretary-general, IX International Botanical Congress, Science Service Building, Ottawa, Ontario, Canada.

In a communication to U. S. botanists from the liaison officer, Dr. Pierre Dansereau (University of Montreal) it was noted:

1. The officers of the Congress are as follows: President, W. P. Thompson, University of Saskatchewan; First vice-president, Pierre Dansereau, University of Montreal; Second vice-president, K. W. Neatby, Science Service, Ottawa; Third vice-president, Muriel V. Roscoe, McGill University, Secretary-general, Clarence Frankton, Science Service, Ottawa; Treasurer, A. J. Skolko, Science Service, Ottawa; Executive secretary, H. L. Berlyn, Science Service, Ottawa; Officer for liaison with U. S. Botanists, Pierre Dansereau; Chairman of Programme Committee, H. A. Senn, Science Service, Ottawa; Chairman of Field Trips Committee, Marcel Raymond, Montreal Botanical Garden; Chairman of Finance Committee, K. W. Neatby; Chairman of Local Organization, Muriel Roscoe; Chairman of Publications Committee, D. L. Bailey, University of Toronto; Chairman of Publicity Committee, H. A. Senn.

2. Responsibility for the Congress rests with Canadian botanists; it is not jointly sponsored by Canadian and U. S. botanists. However, many U. S. botanists have accepted invitations to serve on committees and subcommittees.

3. No special meetings of societies will run concurrently with Congress sectional meetings or be jointly sponsored by them.

INFORMATION AND INSTRUCTIONS FOR CONTRIBUTORS TO PLANT PHYSIOLOGY

I. Submission and Processing of Manuscripts

1. Send the original copy of the manuscript, the first carbon, and two copies of the illustrative material to the Editor, Dr. Allan H. Brown, Department of Botany, University of Minnesota, Minneapolis 14, Minnesota. The author should retain a carbon copy of the manuscript and his original photographs or drawings to insure against loss and to consult in case of need.

2. The Manuscript Will be Sent out for Review by two competent reviewers familiar with the field of research covered by the paper. These reviewers may or may not be members of the Editorial Board. In case of marked disagreement between reviewers, the Editor will appoint a referee, usually a member of the Board, to make a recommendation for the Board. Usually two months suffices for review and decision of the Editorial Board.

3. Manuscripts Returned to the Author for Revision should be sent back to the Editor within two months or the paper will be published under the date of receipt of the revised manuscript and not the original date.

4. Galley Proof will be sent to the author by the printer, but it should be returned directly to the Editor. Correct all errors clearly, and return the galley promptly. Except for corrections of printer's errors, authors will be billed at cost for alterations in the galley proofs involving excessive changes from manuscript copy.

5. Reprint Orders should be sent to the Editor on the form provided along with the corrected galley proof, even if formal requisitions or purchase orders for institutional agencies follow later. Late orders for reprints entail extra work and expense by the printer for which authors must be charged. Since type is destroyed immediately after printing, it is impossible to supply reprints after papers are published.

6. Abstracts for Biological Abstracts are also to be returned to the Editor with the corrected galley. Authors should submit their typed abstracts on the form forwarded with the galley. The Editor will insert proper volume and paging.

II. Preparation of the Typescript

When a manuscript is accepted by the Editor for publication, the Editor and his staff must prepare the typescript for the printer by proofmarking the submitted copy. In proofmarking, the copy is put in a form consistent with the policy of the journal, but at the same time the individual style and personal preferences of the author are retained as far as possible. The author can do much to assure himself that his published articles will be free from typographical and other mechanical errors by carefully preparing his typescript, giving special attention to the form used by the journal, especially in regard to tables, figures, and literature citations.

1. Typing. The typescript must be *double spaced throughout*, including footnotes, figure legends, tables, and literature citations. Only double spaced copy can be proofmarked for the printer, thus eliminating costly corrections. Liberal side margins should also be allowed for proofmarking. *Each table and figure legend and the* Literature Cited *section should be typed* (double spaced) *on a separate piece of paper*, since they are specially set by the printer in a typeform different from that of the body of the manuscript.

2. Length. The allowable length of papers is 10 printed pages, including figures, tables, and literature citations. Authors will be billed at cost for overrun. Approximately four double spaced typewritten pages will equal one page of printed type.

3. Format. Consult recent editions of PLANT PHYSIOLOGY for proper placement of headings, paragraph headings and subheadings, and the use of abbreviations. The processing of manuscripts for publication is expedited and possible sources of serious error are eliminated if the typescript conforms to the established format of published papers.

4. First Page, Footnotes, and Acknowledgments. On the first page of the typescript, above the title, the author should indicate the name and address of the person to whom editorial correspondence, including the galley proof, is to be sent. The name and address of the institution follows the name of the author(s) directly below the title. One or more footnotes are included at the bottom of the first page. The 1st footnote (indicated by superscript 1 after the title) gives the date of receipt of the manuscript or of the revised manuscript (see above); the 2nd footnote credits the source of financial support (if any); and additional footnotes give the address of the author(s), if different from the address in the heading, and indicate the experiment station or institution paper number. Normally these are the only footnotes used. If footnotes must be used in the body of the text, they should be numbered consecutively with Arabic numerals and typed on the bottom of the page in which reference to the footnote is made. Acknowledgments are not included as a footnote, but they are given at the end of the body of the manuscript following the Summary and preceeding the Literature Cited.

5. Underlining. Leave all headings unscored and scientific names without underlining. The Editor will proofmark these items in accordance with the policy of the journal. In general, do not underscore any lines.

6. Numbers. Numerals, cardinal and ordinal, are written out when they begin a sentence. In the body of the text cardinal numbers from one to five are written out except when used with an abbreviation; for example, 2 g, not two g; 4 cm, not four cm. The contracted form is always used for ordinals, i.e., 1st,

10th, 25th. For values of less than one, a zero should be inserted before the decimal point, i.e., 0.7 g, not .7 g. Fractions are normally writen out, and are not hyphenated; for example, one fourth, two thirds, two and one half, except in tables and graphs where 1/4, 2/3, 2 1/2 are more appropriate. It is best, however, to avoid fractions whenever possible and substitute the decimal form, i.e., 0.5, 0.1 and 2.5.

7. TABLES. Authors should study carefully the form of tables used in recent issues of PLANT PHYSIOLOGY and set up their tables in a form consistent with the established policy. Tables should be preplanned to fit one or two printed columns (three inches and six and one-eighth inches wide respectively) and not to exceed eight and one half inches in height, including heading, body and footnotes. Tables may not be rotated 90°. On the average, about 52 pica typewriter spaces fill one line of a three inch column of type of the size normally used in tables, and 10 lines require one inch of the available vertical space. Tables are numbered consecutively in Roman numerals, and footnotes are referred to by the use of the asterisk, double asterisk, dagger, double dagger, and section, in this sequence, as needed. *All explanatory material should be placed in footnotes* rather than in the heading or columnar headings, both of which should be as *brief as possible* without sacrificing clarity.

8. FIGURES. Figures are of two kinds, half-tones and line drawings (including graphs). A very good photograph is necessary for satisfactory reproduction. All photographs should be printed on *glossy paper*, and the print should have good contrast and be in sharp focus, otherwise a grey picture with fuzzy edges may detract from an otherwise excellent paper. Limit the photograph to desired visual details. Trim off irrelevant portions and minimize blank space. The author(s) name, the figure number, and the top of the figure should be indicated on the reverse side of all illustrative material.

All illustrations should be mounted with rubber cement on white cardboard (if a number of pictures are grouped together) or very stiff white paper (suitable for individual pictures) and they should not exceed a total measurement of 9×11 inches to eliminate the necessity of special packaging and handling. Mounting even individual illustrations precludes loss and possible damage from paper-clipping the picture directly to the bulk of the manuscript.

As far as possible all figures should be grouped and mounted close together on white cardboard. Half-tone and line drawings must be placed in separate groups since they are reproduced by different processes. Grouping will result in a considerable saving to the journal, since the minimum engraving charge for the smallest illustration is about one half that for a full page, and for a half page the cost is about three fourths the cost of a full page. When several figures are grouped together, provision should be made on the illustration or in the legend for easy identification by the reader.

The author should plan in advance for a legible finished copy when he is preparing his illustrations. Figures are reproduced the same size as the submitted photograph or they are reduced. The Editor will proofmark the illustrations to determine the actual size of the printed figures, but the author should plan for *maximum reduction*, keeping in mind that the smallest number or letter on the *reduced* illustration should be no smaller than 1 mm to assure legibility. All illustrations must fit either a three inch or six and one eighth inch column and not exceed eight and one half inches in height. If possible, allowance should be made so that the figure legend can be accommodated at the bottom of the printed page. The legend, however, may not be typed on the illustration, but must be typed (double spaced) on a separate piece of paper, since it is set in type by the printer and is not reproduced by the engraver.

Line drawings and graphs should be intensely black on white, and the submitted photographs should be in sharp focus and without blemish. Generally the appearance of a graph is improved if the top and right side is boxed in by lines of the same weight as the abscissa and ordinate. Guided lettering is preferable to hand lettering on figures and graphs. In graphs, special care should be taken to allow for maximum reduction. Symbols, such as triangles, circles and squares can become indistinct upon reproduction if they are not sufficiently large in the original. However, overly large symbols, thick lines or very heavy numerals may also be unsightly or indistinct after reduction.

9. LITERATURE CITED. This section of the typescript requires a great deal of proofmarking for the printer even though it may be correct in every detail. Therefore it is appreciated if authors exercise special care in the preparation of their bibliography. Since the literature citations are set in a typeform different from that used for the body of the manuscript, they should be typed (*double spaced*) on a separate piece of paper. Consult recent issues of PLANT PHYSIOLOGY for the proper form and for abbreviations for journals. Note the limited use of capitals as well as the correct form for citing books and chapters in books. Check to make certain all citations are complete, including title, volume numbers, inclusive pages, and the year of publication. Foreign titles should be rechecked for diaeresis and other markings. The citations should be numbered and listed in alphabetical order (*not* in order of reference) and indicated in the body of the text by Arabic numerals enclosed in parenthesis. Recheck to make certain that all citations are referred to in the text. Unpublished data and personal communications are *not* acceptable as literature citations, but should be referred to parenthetically in the text. Articles that are in press or in preparation may be so designated in the LITERATURE CITED.